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Malnutrition and enteric infections in children in Bengo province, Angola – a four-arm experimental study

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE DOUTOR EM SAÚDE INTERNACIONAL, ESPECIALIDADE EM SAÚDE PÚBLICA TROPICAL

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"Nutrition is both a maker and a marker of development. Improved nutrition is the platform for progress in health, education, employment, empowerment of women and the reduction of poverty and inequality, and can lay the foundation for peaceful, secure and stable societies."

Ban Ki-moon,
United Nations 8th Secretary General,
a message for the SUN Movement Strategy and Roadmap (2016-2020)

RESUMO

Introdução: À semelhança de outros países de baixo-médio rendimento, em Angola, a malnutrição e a doença diarreica estão entre as principais causas de mortes em crianças menores de cinco anos, nomeadamente na província do Bengo.

Objectivos: i) identificar a etiologia da diarreia e fatores associados em crianças menores de cinco anos atendidas no Hospital Geral do Bengo (HGB); ii) fornecer informações sobre a caracterização molecular de rotavírus, antes da introdução da vacina; iii) fornecer uma caracterização molecular de *Giardia lamblia*; e iv) investigar se o tratamento de parasitas intestinais (com ou sem diagnóstico prévio) em dois níveis diferentes (individual ou a nível do agregado familiar) tem impacto sobre o estado nutricional de crianças de 2-5 anos, após seguimento de dois anos no Bengo.

Métodos: Um estudo transversal (ET) foi implementado para investigar a presença de vírus, bactérias e parasitas nas fezes diarreicas de 344 crianças atendidas no HGB (2012-2013), recolhendo dados sociodemográficos, nutricionais e clínicos, explorados por modelos de regressão logística simples e múltipla. Posteriormente, realizaram-se métodos moleculares para identificação dos genótipos circulantes de rotavírus e genótipos e subgenótipos de *G. lamblia.* Entre 2013 e 2017, um estudo longitudinal e experimental (RCT) com quatro braços em paralelo foi realizado em crianças infetadas com pelo menos um parasita intestinal patogénico (ISRCTN-72928001). As 121 crianças com critérios de inclusão foram distribuídas aleatoriamente (1:1:1:1) - Braço1: albendazol anual a nível individual; Braço2: albendazol anual ao agregado familiar; Braço3: diagnóstico e tratamento quadrimestral de parasitas intestinais a nível individual; Braço4: diagnóstico e tratamento quadrimestral de parasitas intestinais ao nível do agregado familiar. No início do estudo, aos 4, 8, 12, 16, 20 e 24 meses de acompanhamento avaliou-se: a altura, o peso, estatura-para-idade, peso-para-altura e peso-para-idade em *Z-score*. A análise por *intenção-de-tratar* foi realizada seguindo as diretrizes CONSORT, após a análise de valores omissos (IBM SPSS). Dada a falha dos pressupostos da análise paramétrica de medidas repetidas, uma abordagem não paramétrica (*npar*LD) e os modelos LMM e GEE foram explorados no programa R.

Resultados: Nos dois estudos, as crianças viviam principalmente em áreas urbanas (>90%) e mais de 20% não tinha latrina. A água mais usada para beber provinha do rio, da torneira no quintal e de tanques. A desnutrição crónica ocorreu em 38% (ET) e 31% (RCT) das crianças. No ET, 67% das crianças estavam infetadas por um agente enteropatogénico, principalmente por *Cryptosporidium* spp. (30%), rotavírus (25%) e *G. lamblia* (22%). *Cryptosporidium* spp. e rotavírus foram mais frequentes em menores de 12 meses. Os principais genótipos circulantes de rotavírus foram: G1P [8] (47%), G1P [6] (29%) e G2P [4] (13%). O genótipo B de *G. lamblia* foi predominante em relação ao genótipo A. No RCT, no início do estudo, as crianças estavam infetadas principalmente com *G. lamblia* (57%) e *Ascaris lumbricoides* (26%). Diferentes modelos não forneceram nenhuma evidência ou fraca evidência do efeito das intervenções nas medições antropométricas, embora tenha ocorrido uma evolução temporal significativa. Contudo, nota-se uma redução da desnutrição ligeira ao longo do estudo, apesar de, em média, as crianças permaneceram com valores padronizados (z-*scores*) negativos para os índices antropométricos.

Conclusão: Várias infeções entéricas foram identificados nos dois estudos. No RCT, nenhuma das estratégias de tratamento de parasitoses intestinais se destacou com efeito significativo nos indicadores antropométricos estudados. A duração do RCT e o tamanho da amostra podem não ter sido suficientes para observar diferenças significativas. Por outro lado, realça-se a importância de uma abordagem multifatorial integrada com vista à melhoria do estado nutricional (*e.g.*, *WASH*, educação, alimentação adequada e acesso a cuidados de saúde)**.**

Palavras-chave: desnutrição, diarreia, desparasitação, crescimento, longitudinal

ABSTRACT

Background: Similar to other low- and middle-income countries, in Angola, malnutrition and diarrhoeal disease are among the major causes of deaths in children under-five, namely in Bengo province.

Aims: i) identify the aetiology of diarrhoea and associated factors in under-five children attending the Bengo General Hospital (HGB); ii) provide information on the molecular characterization of rotavirus, before the vaccine introduction; iii) provide a molecular characterization of *Giardia lamblia*; and iv) investigate if treatment of intestinal parasites (with or without previous diagnosis) in two different levels (individual or household) impacts on nutritional status of children 2-5 years, after a two-year follow-up in Bengo.

Methods: A cross-sectional study (CSS) was conducted to investigate the presence of virus, bacteria and parasites in diarrhoeal stools of 344 children attending HGB (2012-2013), collecting sociodemographic, nutritional and clinical data, analysed by simple and multiple logistic regression models. Then, molecular methods were performed for the identification of rotavirus circulating genotypes and *G. lamblia* assemblages and sub-assemblages. Between 2013 and 2017, a four-arm randomised controlled trial (RCT, registration ISRCTN-72928001) was conducted longitudinally in children infected with at least one pathogenic intestinal parasite. 121 children meeting inclusion criteria were randomly assigned (1:1:1:1) - Arm1: annual albendazole at individual level; Arm2: annual albendazole at household level; Arm3: four-monthly screening and treatment of intestinal parasites at individual level; Arm4: four-monthly screening and treatment of intestinal parasites at household level. Height, weight, height-for-age, weight-for-height, and weight-for-age Z-score were assessed at baseline, 4, 8, 12, 16, 20, and 24 months of follow-up. Intention-to-treat analysis was performed following CONSORT guidelines, after a missing value analysis (IBM SPSS). Given the failure of assumptions for parametric repeated measurements, nonparametric rank-based method (*npar*LD), LMM and GEE models were performed in R program.

Results: In both studies, children lived mainly in urban areas (>90%) and more than 20% did not have a latrine. The most commonly drinking water sources were the river, the tap in the yard and tank. Near 38% (CSS) and 31% (RCT) of children were stunted. In the CSS, 67% of children were infected with an enteropathogen, mostly with *Cryptosporidium* spp. (30%), rotavirus (25%) and *G. lamblia* (22%). *Cryptosporidium* spp. and rotavirus were more frequent in children under 12 months. The main rotavirus circulating genotypes were: G1P[8] (47%), G1P[6] (29%) and G2P[4] (13%). *G. lamblia* assemblage B was predominant compared with assemblage A. In the RCT, at baseline, children were mainly infected with *G. lamblia* (57%) and *Ascaris lumbricoides* (26%). Different models provided no evidence or weak evidence of the effect of interventions on anthropometric measurements, although a significant temporal effect occurred. A reduction in mild malnutrition occurred throughout the study, although, on average, children remained with negative z-scores for anthropometric indices.

Conclusion: Several enteric infections were identified in both studies. In the RCT, none of the treatment strategies targeting intestinal parasites stood out with significant effect on the anthropometric indices studied. The duration of the RCT and the sample size may

not have been sufficient to observe significant differences. On the other hand, it highlights the importance of an integrated multifactorial approach to improving nutritional status (eg, WASH, education, adequate food and access to health care).

Keywords: malnutrition, diarrhoea, deworming, growth, longitudinal.

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LIST OF ABBREVIATIONS

CISA	Centro de Investigação em Saúde de Angola
CDC	Centers for Disease Control and Prevention
EAEC	Enteroaggregative *Escherichia coli*
EDCTP	European & Developing Countries Clinical Trials Partnership
EED	Environmental enteric dysfunction
ETEC	Enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization
GAVI	Global Alliance for Vaccines and Immunizations
GEE	Generalized Estimating Equations
GEMS	Global Enteric Multicenter Study
GOBI	Growth monitoring, Oral rehydration, Breastfeeding and Immunization
MPI	Multidimensional Poverty Index
GNI	Gross National Income
GNMF	Global Nutrition Monitoring Framework
GPS	Global Positioning System
HAZ	Height-for-age Z-score
HDI	Human Development Index
HDSS	Health and Demographic Surveillance System
ID	Iron deficiency
LAZ	Length-for-age Z-score
LMM	Linear Mixed-effect Models
MCAR	Missing completely at random
MDG	Millennium Development Goal
MAL-ED	The Etiology, Risk Factors, and Interactions of Enteric Infections and the Consequences for Child Health and Development Project
MGRS	Multicentre Growth Reference Study
MICS	Multiple Indicator Cluster Survey
MUAC	Mid-upper arm circumference
NCHS	National Center for Health and Statistics
NTD	Neglected Tropical Disease

PCR	Polymerase chain reaction
PoU	Prevalence of undernourishment
PSAC	Pre-school age children
SAM	Severe acute malnutrition
SAGE	Strategic Advisory Group of Experts on Immunization
SD	Standard deviation
SDG	Sustainable Development Goal
SSA	Sub-Saharan Africa
ST-ETEC	*Escherichia coli* producing heat-stable toxin
STH	Soil-transmitted helminths
UHC	Universal Health Coverage
UN	United Nations
UNICEF	United Nations Children´s Fund
USA	United States of America
U5MR	Under-five mortality rate
VAD	Vitamin A deficiency
WASH	Water, sanitation and hygiene
WAZ	Weight-for-age Z-score
WFP	World Food Programme
WFS	World Food Summit
WHO	World Health Organization
WHZ	Weight-for-height Z-score
Z	Z-score

1. INTRODUCTION

In the last decades, the international community has joined efforts to struggle malnutrition, an important public health problem with social and economic impact worldwide (1-9). Special attention has been given to vulnerable groups, including children (2, 5, 10). The normal development of children should be guaranteed, regardless of race, nationality or religion (11).

In 1943, during the United Nations Conference on Food and Agriculture, and two years before the end of the Second World War (1939-1945), it was established the need to secure and adequate food for all (12). There was a scenario of under consumption in the global population, with important impact on several countries, mainly on the child´s health with evident loss in weight and height compared with the pre-war years (13). The goal of freedom from hunger (especially in armed conflict regions), and the need to increase the food production and distribution were important issues to improve (12). Poverty was recognized as the first cause of hunger and malnutrition, which was the leading cause of disease and child mortality (12). Nutrition was a primary concern of governments since it was essential to promote the global economic expansion, to raise the levels of nutrition of people and their life conditions (12).

Following an idea of international cooperation, the United Nations (UN) Charter was formally signed on 26 June 1945 and came into force on 24 October 1945 (14). This was an era marked by the creation of organizations with public health relevance to the present day. The Food and Agriculture Organization (FAO) was founded on 16 October 1945 as a UN specialized agency in order to help countries to find solutions for their food, agriculture and nutrition problems (15). The United Nations Children´s Fund (UNICEF[1]), was created a year later (1946) to help and protect children affected by the war, famine, disease or other type of emergency (16). Two years later, on 7 April 1948, the World Health Organization (WHO) was formally created as an international public health specialized agency of the UN (17), in the same year that the Universal Declaration of

[1] At the time known as The United Nations International Children´s Emergency Fund.

Human Rights was proclaimed and adopted by the UN General Assembly, enhancing the rights and freedoms to which every person is entitled (18).

The 1950s were known as the era of the mass anti-disease campaign through the implementation of water, sanitation and hygiene projects, technical training and nutrition education programmes (16). This decade ended with the UN Declaration of the Rights of the Child (A/RES/14/1386) proclaiming the right to protection, education, healthcare, shelter and good nutrition (19).

In the early 1960s, the World Food Programme (WFP) was established as an initial experimental programme for three years on a voluntary basis to be undertaken jointly by the UN and the FAO (A/RES/1714) (20). The aim of this programme was to establish adequate procedures to respond emergency food needs and emergency in chronic malnutrition, assisting pre-school and school feeding, and promoting the economic and social development through food aid (20).

In the 1970s, there was a recognition that food or *protein crisis* was afflicting especially those living in developing countries (21), leading to the Universal Declaration of the eradication of hunger and malnutrition (A/RES/3348), adopted on 16 November 1974 by the First World Food Conference, proclaiming that *every man, woman and child has the inalienable right to be free from hunger and malnutrition in order to develop fully and maintain their physical and mental faculties* (22). This declaration guided governments to work together to achieve a common goal, through food and nutrition policies, emphasizing the need to *attack on chronic malnutrition and deficiency diseases among the vulnerable and lower income countries* (22). In 1979, the Alma-Ata Conference on Health for All promoted the primary healthcare, which should include, at least, health education, promotion of adequate nutrition, access to safe water and basic sanitation, maternal and child care, immunization, appropriate treatment and control of endemic diseases (23).

During the 1980s, to reduce the under-five deaths, the international community was mobilized to adopt four low-cost techniques, also known as GOBI campaign: Growth monitoring for undernutrition, Oral rehydration to prevent deaths from diarrhoeal disease

Breastfeeding and Immunization[2], which became a priority of the primary health care movement (16). The objective was also to address the control and prevention of infectious diseases in order to improve the nutritional status. During this decade, child nutrition improved globally, although only slightly in sub-Saharan Africa (SSA) (16).

In 1992, commitment to eliminate hunger and reduce all forms of malnutrition was a priority affirmed in Rome, during the First International Conference on Nutrition (24). Poverty and the lack of education continued to be recognized as the main causes of malnutrition and, therefore, the need to improve the access to food, safe water, sanitation, health, education and social services of the most vulnerable was reinforced (24, 25). Concerns were focused on Africa, Asia and Latin America, where the number of malnourished children was increasing and the infectious, parasitic and communicable diseases needed to be reduced through adequate health care services and national programs (25, 26). The Rome declaration, adopted in 1996 during the World Food Summit (WFS), also listed commitment points for achieving food security at the individual, household, national, regional and global level (27). International cooperation continued, but each country was requested to participate by formulating its own goals and strategies.

In 2000, the UN Millennium Declaration was the basis of the eight international Millennium Development Goals (MDGs) established to be achieved by 2015 (28). The first goal (MDG 1) aimed to *eradicate extreme poverty and hunger,* which included the target 1c of reducing by 50% the proportion of undernourished people who suffer from hunger between 1990 and 2015 (29). Two main indicators were used to assess the progress of MDG nutrition targets: the proportion of undernourished people and the prevalence of underweight in children under-five years of age (29, 30). The nutrition targets were integrated in many international initiatives to inspire and to promote a global movement towards nutrition challenges (2, 3, 5, 24, 30-32).

[2] Immunization against the six vaccine-preventable childhood killers: tuberculosis, diphtheria, whooping cough, tetanus, polio and measles

INTRODUCTION

After the MDGs era (33), the list of the 17 Sustainable Development Goals (SDGs)[3] represents now a new call to *reducing health inequalities and leaving no one behind until 2030* through a multisector approach set by the UN (6, 34-36). The second SDG aims to *end all forms of malnutrition* by 2030 (Zero Hunger), by achieving also the six global nutrition targets[4] by 2025, set by the World Health Assembly in 2012, and integrated in a comprehensive implementation plan on maternal, infant and young child nutrition (34, 35). Reducing by 40% the number of stunted children under-five years of age, and wasting to less than 5% are one of the targets to be achieved (34, 35).

Recent data indicates that in 2017 approximately 151 million (22.2%) children under-five years of age were stunted, 50.5 million (7.5%) were suffering from wasting, and 38.3 million (5.6%) were overweight, worldwide (37). Malnutrition contributes as an underlying cause with 45% of total deaths in this age group (38) and, simultaneously, infectious diseases are the major, although preventable, causes of death in developing regions (1).

Malnutrition and infectious diseases are a serious health problem in Angola, a sub-Saharan country that faced a 40-year period of civil war until 2002 (39). According to the most recent Multiple Indicator Cluster Survey (MICS) conducted in 2015-2016, 37.6% of Angolan children are suffering from stunting and 4.9% from wasting (40). There is a national commitment included in the National Health Development Plan to struggle malnutrition and, consequently, to prevent and treat its associated diseases (41). Some of the strategies include the promotion of adequate breastfeeding practices, micronutrient supplementation, vaccination (mainly by introducing rotavirus vaccine in the national immunization plan), and deworming with albendazole in under-five children (41, 42).

Bengo province is one of the eighteenth provinces of Angola, where malnutrition and infectious diseases are also a major problem in children (40, 43). This thesis seeks to contribute to literature on malnutrition and enteric infections with data collected from a

[3] Annexe I – List of the 17 Sustainable Development Goals

[4] Annexe II – Goal 2 - Zero Hunger: Targets and indicators

cross-sectional hospital-based study and a longitudinal study conducted in Bengo. The first study provides a global characterization of enteric infections (virus, parasites, bacteria) in under-five children with diarrhoea and its association with malnutrition, by answering the following questions: i) what are the most frequent agents of diarrhoea in children attending the referral hospital for the province?; ii) what is detection rate of rotavirus and circulating genotypes before the introduction of rotarix vaccine?, and iii) what is the detection rate and molecular profile of *Giardia lamblia*? Then, restricting our focus on undernutrition and intestinal parasitic infections, a four-arm parallel trial was implemented to investigate: iv) if the treatment of intestinal parasites (with or without previous diagnosis) in two different levels (individual or household) impacts on nutritional status of children between two and five years old, after a two-year follow-up period.

This work is divided in three main sections: Introduction, Results and Discussion.

The first section begins with a brief definition of malnutrition (1.1) and global prevalence rates (1.2). Then, based on the UNICEF conceptual framework, the main factors affecting the nutritional status are described and contextualized within the Sustainable Development Goals (1.3), as well as international strategies to improve child nutrition (1.4). After, the profile of Angola is presented considering important historical facts, the health system, its main indicators and nutrition targets to be achieved in the context of the SDGs (1.5). The introduction closes with the main objectives of this thesis (1.6), as well as the methodology applied in the different studies performed (1.7), and all references used in this first section (1.8).

The second section is the Results section, which is composed by four papers of which three are already published and one was submitted to be published: paper I (2.1), paper II (2.2); paper III (2.3) and paper IV (2.4).

The third section is the Discussion, where the main findings are discussed from a public health perspective, with an overview of the research limitations, strengthens, conclusions, and recommendations for future research in this field.

1.1. Defining Malnutrition

Malnutrition encompasses two groups of conditions: undernutrition and overnutrition (2). Undernutrition is related to undernourishment, poor absorption or deficit of nutrients intake (44). It includes stunting, wasting, underweight and deficiency of micronutrients (vitamins and minerals), which can appear isolated or in combination. Overnutrition, in turn, is a consequence of over-consumption of nutrients and can lead to overweight and obesity (5, 44). The co-existence of undernutrition and overnutrition across the life course is known as *double burden of malnutrition* (45). In this thesis, undernutrition is our main focus.

1.1.1. Anthropometric indices to assess growth in children

Child malnutrition can be identified through different methods such as anthropometric measurements, biochemical markers, clinical assessment (identification of bilateral oedema and gastrointestinal symptoms) and dietary methods (46, 47). Since it is a non-invasive and a less expensive method, anthropometric assessment has been largely used to assess growth in children under-five, especially in developing countries (47-49).

Growth reflects the welfare, quality of life of the individuals and, therefore, can be used to predict health and survival of populations (44, 50, 51). To compare measurements obtained from surveys conducted in different regions, or in the same region but at different moments, it is essential the use of an international reference[5] (52). Thus, in 1977, the WHO recommended the use of the National Center for Health and Statistics (NCHS) growth curve of the Centers for Disease Control and Prevention (CDC), also known as NCHS/WHO international reference population (50, 52). Over the years, the NCHS/WHO growth reference played as an important guide for screening and monitoring malnutrition worldwide (50, 52). However, several technical and scientific limitations

[5] The WHO Expert Committee defined reference as a toll for grouping and analysing data through a common basis for comparing populations, but not for making inferences about the meaning of observed differences.

were pointed out to these curves (48, 53-56): 1) they were developed on data collected in only one country (the USA), and for that reason it was not representative of children´s growth patterns from different regions of the world (56); 2) the distribution of weight in the reference population was positively skewed, with a high prevalence of overweight, resulting in a non-healthy sample (56); 3) the growth curves were a result of two distinct data sets collected in different moments (1929-1975 for children under 24 months of age, and 1960-1975 in the children with 2-18 years), while a single sample would be the ideal (50, 56); 4) and the dataset reflected the growth of formula-fed infants, rather than breastfed infants (56).

Therefore, in 1993 the WHO concluded that it was necessary to create new curves which could represent more adequately the growth of children (54, 55). Under this perspective, between 1997 and 2003, the Multicentre Growth Reference Study (MGRS) was conducted and data from approximately 8.500 healthy children from different regions of the world (Brazil, Ghana, India, Norway, Oman and the USA) was collected, combining a longitudinal follow-up (0-24 months) and a cross-sectional survey (18-71 months) (54). The MGRS provided standards describing how children should grow under optimal conditions (54), including adequate feeding practices (breastfeeding established as the standard for measuring healthy growth), good healthcare and environmental conditions (infants with morbidity and infants whose mothers did not follow the recommended exclusive breastfeeding and non-smoking guidelines were excluded) (54, 57). This study confirmed that the effect of ethnic differences on the growth of children is small compared with the effects of environment, including infectious diseases, inadequate diet, and poverty (46, 58).

Thus, in April 2006 the MGRS led to the launch of the WHO Child Growth Standards, also known as WHO standards (54). Since then, many countries adopted and implemented the Z-score system of WHO standards in their nutrition and public health sectors (54, 59). Switching from NCHS/WHO curves to WHO standards brought some differences described elsewhere (54, 60).

There are three basic measurements in anthropometry: age, weight and height (or length in children under two)[6] (48). Anthropometric indices result from a combination of measurements and enables its interpretation (48). They can be expressed in Z-scores, percentiles[7], or percent of median (51). Z-scores can be used as continuous variables with a normal distribution (*Gaussin distribution*) with mean equal to zero and standard deviation (SD) equal to 1 (Figure 1), and it represents the deviation of the value for an individual from the median or mean value of the reference, divided by the standard deviation for the reference population (61, 62), Figure 1. Z-scores are more useful for research, whereas percentiles are easier for use in clinical settings (62).

Figure 1. Cumulative probability and percentile of Z-score

For normal distribution (*Gaussian distribution*), Z-scores and percentiles are mathematically related (57). A Z-score of 0 divides the total area into equal halves. Z-scores of 1, 2 and 3 are found to the right, and -1, -2 and -3 to the left. Each point of Z-score corresponds to a percentile. The commonly used -3, -2 and -1 are, respectively, 0.13th, 2.28th, and 15.8th percentiles. The Z-score of 1 corresponds to the 84th percentile (0.5+0.34), i.e., 84% of the population are measured lower than Z-score of 1, and a Z-score of 2 corresponds 97.7th percentile. A Z-score is calculated as dividing the difference between value measured (x) and the mean (μ) by standard deviation (σ). Adapted from (63).

The most common anthropometric indices are height-for-age (HAZ), weight-for-length/height (WHZ) and weight-for-age Z-score (WAZ) (49, 51, 54)[8].

[6] For more detail on the assessment of weight and length/height in children see Annexe III.
[7] A percentile is the value below which a certain percentage of observation falls. The most common percentiles include the 3rd, 5th, 50th (median), 85th, 95th, 97th and 99th.
[8] For simplification, the term height is used throughout the thesis. However, the reader should be aware that in children below two years (or less than 85 cm), the term length is used instead of height: length-for-age (LAZ) or weight-for-length. Length refers to the measurement in recumbent position, whereas stature refers to standing height measurement. For more detail see Annexe III.

The use of Z-score is widely recommended since, as standardized measures, they are comparable across different groups (e.g., sex and age groups). Thus, calculating a Z-score is useful for comparing a child with a reference population. As an example, in Figure 2, Z-score lines are numbered positively (1, 2, 3) or negatively (-1, -2, -3), and according to the point plotted in the graphical representation, it is possible to see that the girl is 4 years and 6 months of age and measures 106 cm of height. Her height-for-age is plotted in the median, with a Z-score between -1 and 1, which means that she is in the normal range (51, 57).

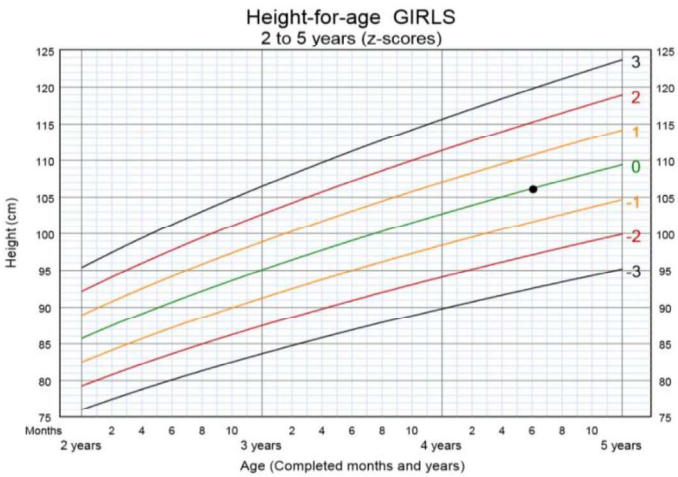

Figure 2. Graphical representation of height-for-age of a girl

The horizontal reference line (x-axis) represents the age (in years and months) and the vertical reference line (y-axis) shows the height (cm). Source: (64)

Therefore, depending on the Z-score obtained and by using some cut-offs, different levels of malnutrition can be identified in each one of the anthropometric indices: mild, moderate and severe, Table 1 (44, 54).

Table 1. Anthropometric indices to assess growth in children

Anthropometric indices	Indicator	Interpretation	Degree of malnutrition	Cut-off value
HAZ	Stunting	Poor nutrition and recurrent infections	Mild	-2 ≤ Z-score < -1
			Moderate	-3 ≤ Z-score < -2
			Severe	Z-score < -3
WHZ	Wasting	Weight loss due to recent episode of infection or inadequate nutrient intake	Mild	-2 ≤ Z-score < -1
			Moderate	-3 ≤ Z-score < -2
			Severe	Z-score < -3
WAZ	Underweight	Can be a result of acute or chronic malnutrition	Mild	-2 ≤ Z-score < -1
			Moderate	-3 ≤ Z-score < -2
			Severe	Z-score < -3

HAZ: height-for-age Z-score; WHZ: weight-for-height Z-score; WAZ: weight-for-age Z-score. Adapted from (54)

According to 2006 WHO Child Growth Standards, a child is considered stunted, wasted or underweight when the Z-score is below -2 (including the moderate and severe forms) for HAZ, WHZ and WAZ, respectively (49, 54) – Table 1. When the Z-score of a child is located between -1 and 1, it means that she or he is growing and developing normally for the indicator assessed, as demonstrated previously in Figure 2.

Underweight (assessed by WAZ, Table 1) was the most used indicator to assess the progress of nutrition targets during the MDG era (29, 30). Despite being easy to assess weight, interpretation of underweight is difficult, as it can be a result of acute or chronic malnutrition (4, 44, 54). Therefore, to assess malnutrition it is more useful to consider wasting and stunting instead of underweight (65).

A new emphasis have emerged on stunting in recent years, especially after being endorsed as a key indicator for monitoring infant and young child nutrition during the Sixty-sixth World Health Assembly in 2012 (9, 34, 35, 66, 67). Stunting, or chronic malnutrition (assessed by HAZ, Table 1), is a result of a long term process, often beginning in utero (as a consequence of the mother´s malnutrition) and is usually associated to recurrent nutrition deprivation and infections during the childhood (6, 44). It is an indicator of linear growth retardation and a barrier to improvements on child health, educational performance and economic development (44, 68).

Wasting (thinness, assessed by WHZ, Table 1) is a symptom of acute malnutrition and results from immediate and inadequate nutrient intake or infection associated, mainly diarrhoea, leading to a rapid weight loss (54, 68). Severe acute malnutrition (SAM) can be assessed by WHZ status (Z-score<-3) or by using mid-upper-arm circumference (MUAC) criteria for children between 6 and 59 months of age (MUAC<115mm, with or without oedema) (46, 69). MUAC was introduced for the assessment and management of SAM in the community (70) to identify high-risk malnourished children (71). Kwashiorkor and marasmus are forms of SAM, which can be identified through clinical assessment (46). Kwashiorkor (oedematous malnutrition) is characterized by the presence of oedema, the muscles are wasted, and the hair is usually thin, sparse and discoloured (72, 73). In Marasmus (non-oedematous malnutrition) the child does not present oedema and there is a loss of muscle and fatty tissue (74). Some children can have signs of both kwashiorkor and marasmus, which is known as marasmic-kwashiorkor (74). Children who are severely malnourished have a high risk of death exceeding 9-fold that of children with a Z-score > -1 (2, 46).

1.1.2. Micronutrient deficiencies

Poor quality diets can contribute micronutrient deficiencies, also known as *hidden hunger*. Deficiency of micronutrients, such as vitamin A, iron, zinc and iodine, among others, can lead to important health consequences, since they are essential to the physical and mental development (5, 75).

Iron deficiency (ID) is the major cause of anaemia and results from inadequate intake or absorption of iron (76). Anaemia is a condition in which the oxygen-carrying capacity of red blood cells is compromised and develops especially in periods of life in which iron requirements are higher, such as during pregnancy and infancy (77). Malaria and helminth infections can also lead to anaemia, especially in children living in endemic regions (78, 79). During the childhood, iron deficiency can result in fatigue, reduced physical capacity, and contributes to impaired growth since it is required for development and cell growth of the immune and neural systems, metabolism and exercise (78).

Anaemia can be classified through the assessment of blood haemoglobin concentration, as presented in Table 2. The diagnosis of anaemia can be also performed through serum

ferritin and/or transferrin receptor, but in poor-resource settings the diagnosis based on clinical signs (palmar pallor in children) and medical history is commonly used (77).

Table 2. Classification of anaemia according to haemoglobin concentration

Diagnosis of anaemia (6-59 months of age)	**Haemoglobin concentration** (g/L)
No anaemia	≥ 110
Mild anaemia	100-109
Moderate anaemia	70-99
Severe anaemia	< 70

Adapted from (77)

Vitamin A deficiency (VAD) has been associated to increasing number of deaths from measles and diarrhoea, as well as higher severity of respiratory illness and susceptibility to malaria (2, 75, 80). Children are the age group at higher risk of enteric infections, which can contribute to reducing the vitamin A absorption (81). This micronutrient is essential to the normal functioning of the visual and immunity systems, epithelial integrity, red blood cell production, reproduction and growth (80). VAD is defined as a serum retinol concentration < 0·70 μmol/L, and can lead to several ocular manifestations known as xerophthalmia (including Bitot spots), which can progress to blindness (5, 80, 81).

Another important micronutrient is iodine, especially for the synthesis of thyroid hormone, which is essential for neuronal migration and myelination of the fetal brain (44, 82, 83). In some regions, the soils have a poor iodine concentration and, consequently, the food grown in these soils are also deficient in iodine. Thus, when there is a deficit consume of iodine, the thyroid is not able to synthesize sufficient amount of thyroid hormone, which can contribute to iodine-deficiency disorders (84, 85). One of the most important consequences of this disorder is the brain damage (which can occur even before birth), and impaired cognitive development in children (82, 84).

Since more than 90% of dietary iodine appears in the urine, the urinary iodine concentration is used as biomarker of recent iodine intake (86). According to WHO, school-aged children should intake 90 μg of iodine daily, and severity of iodine deficiency

in children can be defined as a median urinary iodine concentration of under 100 μg/l, as presented in Table 3 (85).

Table 3. Classification of iodine deficiency level in children

Iodine deficiency	Median urinary iodine concentration (μg/l)
No	≥ 100
Mild	50-99
Moderate	20-49
Severe	< 20

Adapted from (44)

Zinc is essential for the immune system, cell division, and growth from in utero until puberty (75). Its deficiency can result from deficient diet in zinc or morphological and functional changes in the small intestine (excessive intestinal loss of zinc and impairment in small intestine absorptive function), and has been associated to poor health, increased risk of diarrhoea and pneumonia, and impaired growth (5, 87-89). However, zinc supplementation has been only recommended in the clinical management of acute diarrhoea (along with other strategies) by giving 20 mg per day of zinc supplementation for 10-14 days (10 mg per day in infants under six months) (44, 90, 91).

1.2. Global prevalence of malnutrition

1.2.1. Stunting, wasting and overweight

There are well-defined prevalence thresholds for stunting, wasting and overweight (Table 4), which are important for identifying priority countries for action and for monitoring progress towards nutrition targets to be achieved by 2025 (92).

Table 4. Prevalence thresholds for stunting, wasting and overweight in children under-five

Labels	Prevalence thresholds (%)		
	Stunting	Wasting	Overweight
Very low	< 2.5	< 2.5	< 2.5
Low	2.5 – < 10	2.5 – < 5	2.5 – < 5
Medium	10 – < 20	5 – < 10	5 – < 10
High	20 – < 30	10 – < 15	10 – < 15
Very high	≥ 30	≥ 15	≥ 15

Adapted from (92)

The global proportion and the number of children under-five estimated to suffer from stunting, wasting and overweight in 2000 and 2017 are presented in Figure 3 (37).

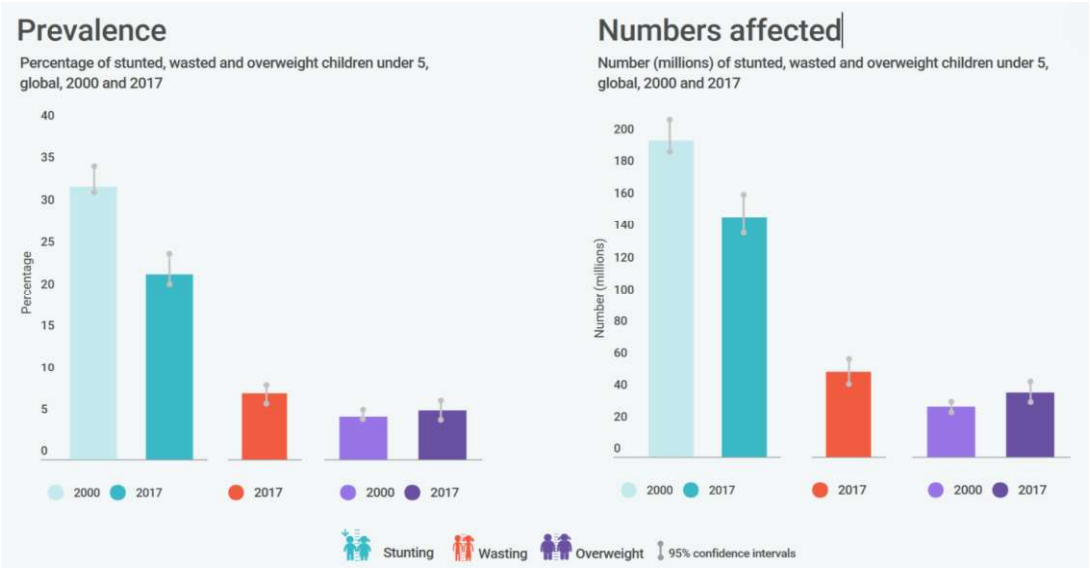

Figure 3. Global prevalence and number of children with stunting, wasting and overweight in 2000 and 2017
Source: (93)

The graph shows that the global prevalence of stunting reduced from 32.6% (very high level in 2000) to 22.2% (high level in 2017); while overweight prevalence increased from 4.9% (in 2000) to 5.6% (in 2017); and 7.5% of children were suffering from wasting in 2017. The global number of children affected by stunting also decreased in the same period (from 198 million to 151 million), while those with overweight increased (from 30.1 to 38.3 million). The same data are also described in the Table 5, but by regions and sub-regions of the world (94).

Table 5. Progress of stunting, wasting and overweight in under-five children by regions and sub-regions of the world

	Stunting[1]				**Wasting**[1,2]		**Overweight**[1]			
	2000		2017		2017		2000		2017	
	n	(%)	n	(%)	n	%	n	(%)	n	(%)
World	**198.4**	**(32.6)**	**150.8**	**(22.2)**	**50.5**	**(7.5)**	**30.1**	**(4.9)**	**38.3**	**(5.6)**
Africa	**50.6**	**(38.3)**	**58.7**	**(30.3)**	**13.8**	**(7.1)**	**6.6**	**(5.0)**	**9.7**	**(5.0)**
Eastern Africa	21.5	(45.7)	23.9	(35.6)	4.0	(6.0)	2.2	(4.7)	3.0	(4.4)
Middle Africa	7.1	(40.2)	9.3	(32.1)	2.1	(7.1)	0.8	(4.4)	1.4	(4.7)
Northern Africa	4.9	(23.8)	5.0	(17.3)	2.3	(8.1)	1.7	(8.2)	3.0	(10.3)
Southern Africa	2.0	(33.1)	2.0	(29.1)	0.3	(4.0)	0.6	(10.3)	0.9	(13.7)
Western Africa	15.1	(36.9)	18.6	(29.9)	5.1	(8.1)	1.3	(3.2)	1.5	(2.4)
Asia*	**134.6**	**(38.1)**	**83.6**	**(23.2)**	**35.0**	**(9.7)**	**13.9**	**(3.9)**	**17.5**	**(4.8)**
Central Asia	1.6	(28.0)	0.9	(11.8)	0.3	(3.7)	0.5	(8.9)	0.8	(10.7)
Eastern Asia	17.1	(19.2)	4.8	(5.3)	1.7	(1.8)	5.5	(6.2)	4.8	(5.2)
Southern Asia	89.5	(49.6)	58.7	(33.3)	26.9	(15.3)	4.6	(2.5)	5.4	(3.1)
South-eastern Asia	21.0	(38.4)	14.9	(25.7)	5.1	(8.7)	1.7	(3.2)	4.2	(7.3)
Western Asia	5.4	(23.3)	4.2	(15.2)	1.1	(3.9)	1.5	(6.7)	2.3	(8.2)
Latin America and the Caribbean	**9.7**	**(16.9)**	**5.1**	**(9.6)**	**0.7**	**(1.3)**	**3.9**	**(6.8)**	**3.9**	**(7.3)**
Caribbean	0.6	(15.0)	0.3	(8.0)	0.1	(3.2)	0.2	(5.3)	0.3	(7.2)
Central America	4.1	(23.9)	2.3	(14.1)	0.1	(0.9)	1.0	(5.7)	1.0	(6.4)
South America	5.0	(13.8)	2.5	(7.5)	0.4	(1.3)	2.7	(7.5)	2.6	(7.7)
Oceania**	**0.4**	**(36.8)**	**0.5**	**(38.1)**	**0.1**	**(9.2)**	**0.1**	**(4.7)**	**0.1**	**(7.9)**

[1] Includes moderate and severe forms (Z-score<-2); [2] Only recent trends are presented for wasting due to rapid change that this acute condition can suffer over the course of a year period. *Asia and Eastern Asia excluding Japan; **Oceania excluding Australia and New Zealand. Adapted from (94)

Stunting rates dropped in all regions: Africa from 38.3% to 30.3%, Asia from 38.1% to 23.2%, the Latin America and the Caribbean from 16.9% to 9.6%, with the exception of Oceania, increasing from 36.8 to 38.1% (94), Table 5. The number of stunted children

declined in most regions, but in Africa it rose, probably due to the population increase (5, 35). Despite the improvements, stunting continues to be an important public health problem, especially in Africa and Asia (37), with considerable variation within sub-regions, Table 5.

The great number of stunted children was in Asia (almost 70.0%) and Africa (slightly more than 27%), Table 5. The proportion of wasted children was higher in Asia (9.7%), compared to other regions such as Africa (7.1%), Latin America and the Caribbean (1.3%) and Oceania (9.2%) (94).

Unlike wasting and stunting, the global proportion of overweight increased over the years, as it was previously mentioned. This trend was noted in all regions as a whole (with the highest increase registered in Oceania), with the exception of Africa, where the prevalence remained constant (5.0%), Table 5.

1.2.2. Micronutrient deficiencies

Over two billion people are suffering from micronutrient deficiencies (95). Iron deficiency is the one affecting the highest percentage of children under-five years of age worldwide, accounting for 42% (77). The proportion of anaemia attributable to iron deficiency for preschool children is 28% for Sub-Saharan countries and 24% for South-East Asia. The magnitude of anaemia at country-level can be classified as a public health problem according to its prevalence, as describe in Table 6 (77).

Table 6. Classification of anaemia as a problem of public health significance

Prevalence of anaemia (%)	Category of public health significance
< 4.9	No public health problem
5.0 – 19.9	Mild public health problem
20.0 – 39.9	Moderate public health problem
≥ 40	Severe public health problem

Adapted from (77)

Trends and mortality effects of vitamin A deficiency in children was provided by a pooled analysis of population-based surveys from 138 low-income and middle-income countries (96). The analysis showed that between 1991 and 2013 the prevalence of VAD fell from 39% to 29%, respectively. Despite the global progress, there has been no evidence of decreasing in the prevalence of vitamin in SSA and South Asia. Indeed, these were the regions with the highest prevalence of VAD in 2013: 48.0% for SSA (ranging from 25 and 75%) and 44.0% for South Asia (13-79%). Nearly 1.7% of all deaths in under-five children were attributable to VAD in 2013, of which more than 95% occurred in SSA and South Asia (96).

Estimates from 2011 indicate that the iodine intake was insufficient for (<100 µg/l) 29.8% of school-aged children worldwide (5.2% with severe, 8.1% with moderate, and 15.9% with mild iodine deficiency) (86). Off the total children with insufficient iodine intake (240.9 million), 31.5% and 24.1% in southeast Asia and Africa, respectively. Iodine intake was estimated to be insufficient in 32 countries, where Angola was included and categorized as moderately deficient, as shown in Figure 4.

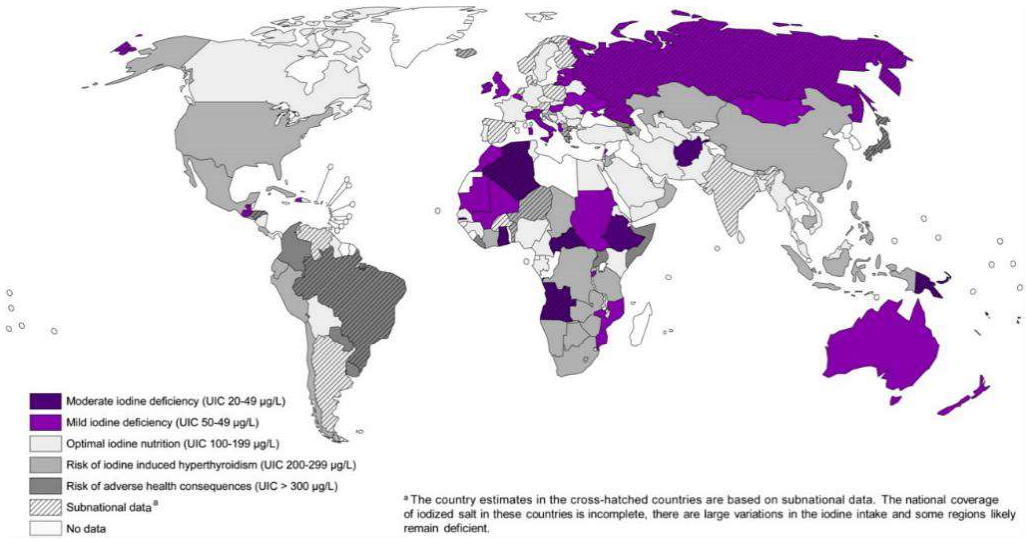

Figure 4. Degree of public health importance of iodine deficiency based on urinary iodine concentration in school-age children in 2011.
Source: (86)

According to global estimates, 17.3% of the world´s population were at risk of inadequate zinc intake during 2003-2007, especially in SSA and south Asia and in regions, as shown in Figure 5 (97). The estimates found that the higher the percentage of dietary zinc obtained from foods of animal origin, the lower the prevalence of inadequate zinc intake (97).

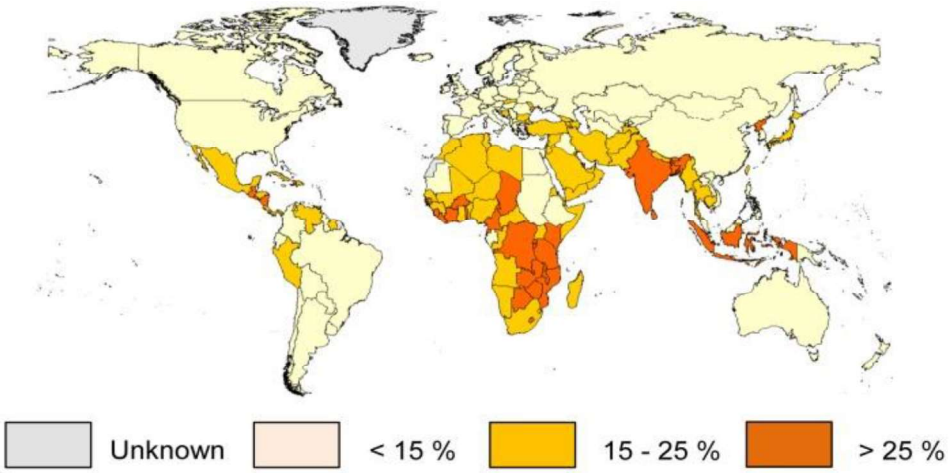

Figure 5. Prevalence of inadequate zinc intake (2003-2007)
Source: (97)

1.3. Conceptual framework on malnutrition and the Sustainable Development Goals

The UNICEF conceptual framework on nutrition, originally created in 1990, has been used to emphasized the complexity of malnutrition, and to identify priority areas for action in this sector during the last decades (2, 25). Currently, the international community is joining efforts to *reducing health inequalities and leaving no one behind until 2030* through the SDGs (36)[9]. Considering the multifactorial causality of malnutrition, it is understandable that achieving all SDGs is a way to improve nutrition and, at the same time, achieving nutrition targets is a key to the success of SDGs. Thus, in Figure 6 it is represented not only the potential causes of malnutrition and its associated short- and long-term consequences (Figure 6-A), as well as some of the SDGs targets and indicators that play an important role in this field (Figure 6-B) (2, 25).

Children suffering from chronic malnutrition in the early stages of life, especially during the first 1000 days after conception, can fail to achieve their potential growth (2, 5, 98, 99) and, consequently, they have an increased risk of chronic diseases in adulthood and reduced productivity, with impact not only at the individual level, but also at the household and national levels (2, 3, 6, 9, 100). Specifically, the framework identifies three main groups of causes of undernutrition: immediate, underlying and basic causes (Figure 6-A). The immediate causes are linked with individual factors such as the inadequate dietary intake and disease, and in most cases it is a combined result of both (2, 5, 25, 101). The underlying causes are those that can influence the bidirectional cycle between nutrition and disease at the community level, such as the household food insecurity, the inadequate care and feeding practices, unhealthy household and environment (poor drinking water, sanitation and hygiene), as well as poor access to health services (98, 102). The basic causes represent the global context of certain region or country, including the social cultural and political factors, policies and programs adopted that can influence the access to essential services and resources (102, 103). All these factors together can influence the economic growth and health outcomes and, consequently, are strictly related to poverty (2, 3).

[9] Annexe I - List of the 17 Sustainable Development Goals

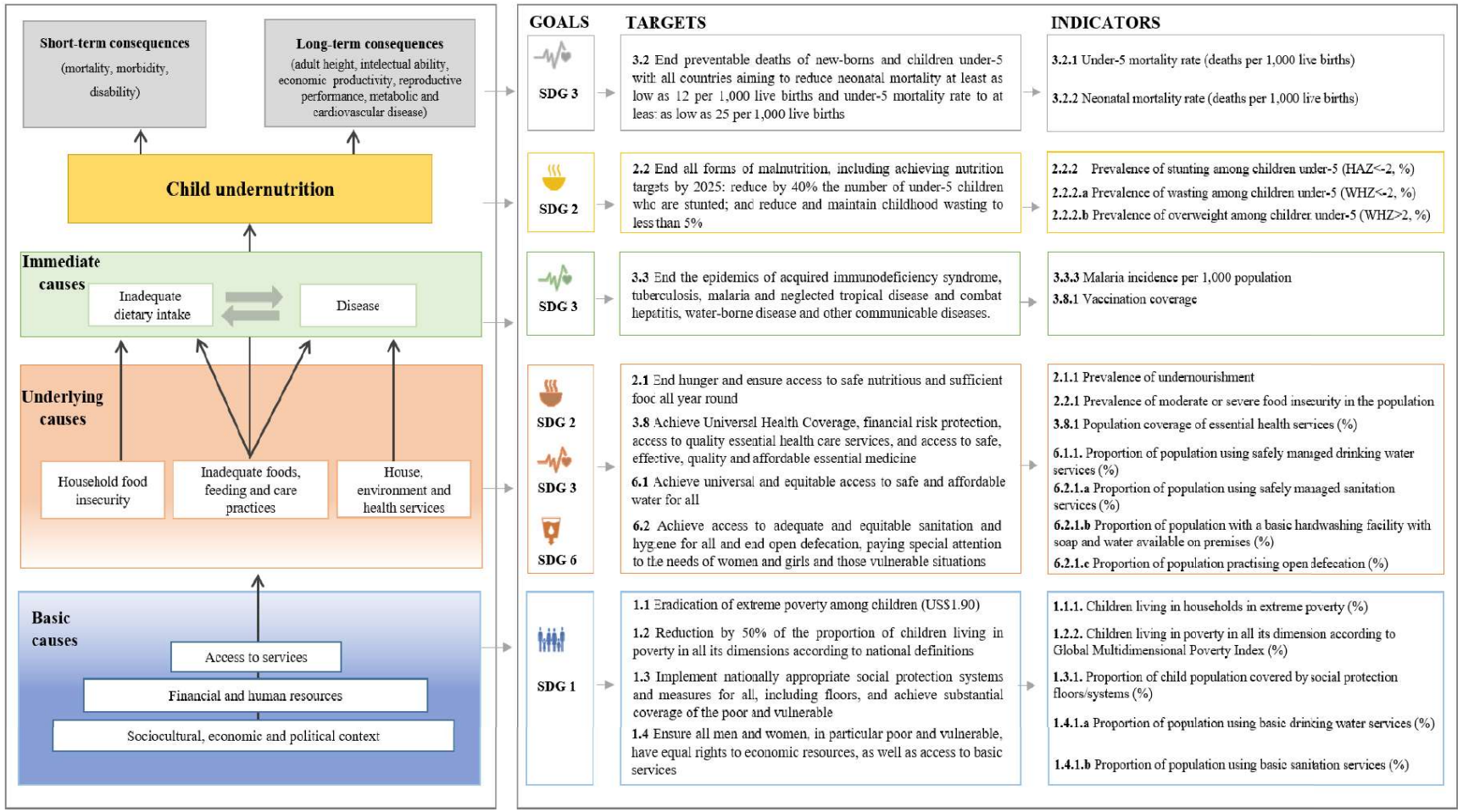

Figure 6. Conceptual framework of malnutrition and its association with the SDGs
A – UNICEF Conceptual Framework on malnutrition; **B –** SDGs: Goals, targets and indicators. Under-5: under five years of age. SDG: Sustainable Development Goal. Adapted from: (25, 36)

1.3.1. Malnutrition and infection

The inadequate intake of nutrients (deficiency, excess or imbalance of nutrients) and disease (affecting the digestion, absorption, transport and utilization of nutrients) are factors (Figure 6-A) with impact on the body structure (such as weight and height) and functions, on the psychosocial well-being, and on the individual clinical outcome (2, 5, 25). For this reason the inadequate dietary intake and disease are identified as the immediate causes of malnutrition (2, 5, 25, 101).

The vicious cycle between malnutrition and infection was emphasized by Scrimshaw in the monograph entitled *Interactions of Nutrition and Infection* (1968), based on two basic patterns: *malnutrition generally alters resistance of the host to infection, and infectious diseases exaggerates existing malnutrition* (104). This means that infection can be, at the same time, the cause and the consequence of malnutrition (104). Meanwhile, an additional key concept was recently added to this cycle: the environmental enteric dysfunction (EED) as an important mediator between the child and the acquisition of nutrients necessary for growth (71, 105), as shown in Figure 7.

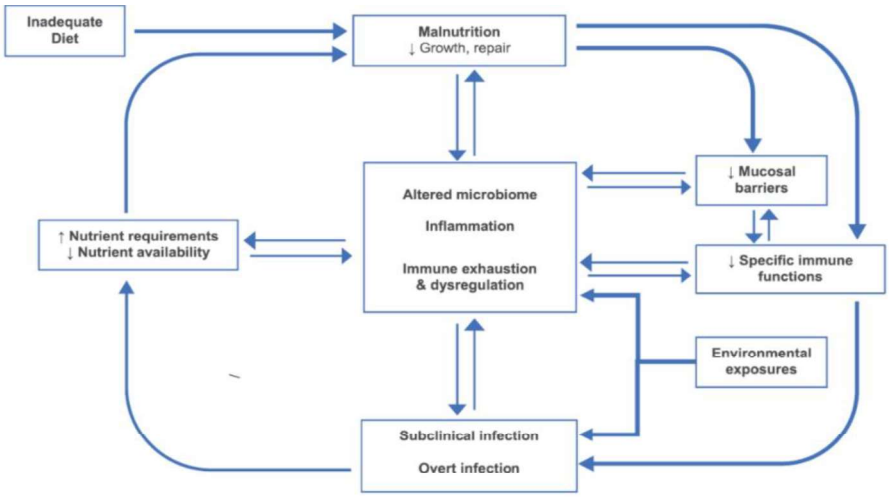

Figure 7. Malnutrition, infection and intestinal dysfunction

Malnourished children can be more susceptibility to infection, which, in turn, may lead to reduced nutrient absorption and nutrient losses (↓*mucosal barriers*), diversion of nutrients to inflammatory responses (*inflammation,* ↑ *nutrient requirements*) and tissue repair (104-106). The circulating levels of vitamin A, zinc suffer a decrease, and iron remains in the liver, restricting its availability to other tissues of the body *(*↓ *nutrient availability*) and, consequently, the nutritional status deteriorates via anorexia (↓ *growth*) (96). EED affects mostly children who are repeatedly exposed to faecal contaminated environments (*environmental exposures)* that induces morphological changes in the small intestine (*altered microbiome*) (105, 106). It is characterized by an asymptomatic syndrome (*subclinical infection*) of poor absorption, local intestinal inflammation (*inflammation)* and systemic immune activation (*immune exhaustion & dysregulation).* Source: (71).

EED syndrome has been associated to stunting and linked with the reduced efficacy of oral vaccines in developing countries, although the exact mechanisms are not clearly understood and further research is needed (106, 107).

The vicious cycle between malnutrition and infection have relevant impact on the morbidity and mortality of vulnerable groups (100, 104, 105, 108-111). Children severely malnourished are at higher risk of infectious diseases, with higher incidence and severity (71, 112). Acute diarrhoeal disease and respiratory infections are the most frequent infections during the childhood in low- and middle-income countries (112, 113), but only the former will be addressed in this thesis.

1.3.1.1. The magnitude of diarrhoeal disease

According to the WHO definition, diarrhoea is the passage of three or more loose or liquid stools per day (or more frequent passage than is normal for the individual) (114). Acute watery diarrhoea can last for several hours or days, while persistent diarrhoea lasts 14 days or more. The presence of blood in diarrhoeal stool (acute bloody diarrhoea) is also known as dysentery (114).

Diarrhoeal disease can lead to rapid fluid loss, poor absorption of nutrients and marked loss of proteins, vitamin A, zinc and other micronutrients. In a healthy child, an episode of diarrhoea is usually self-limiting with no long-term consequences. However, in children living in poor settings, if the fluids are not replaced, chronic diarrhoea can lead to severe dehydration, malnutrition and death (113).

The most common mode of transmission of the pathogenic agents causing diarrhoea (virus, parasites and bacteria) is faecal-oral transmission: by ingesting water or food contaminated with the stool of a person infected (114). The leading risk factors for diarrhoea deaths described in the Global Burden of Disease Study 2016, a systematic analysis including 195 countries, were childhood wasting (80.4%), unsafe water (72.1%) and unsafe sanitation (56.4%) (112). Chronic diarrhoea is also a common symptom among the clinical syndrome of acquired immune deficiency syndrome (113).

Diarrhoea is the fifth leading cause of death in children younger than five years (8.9% of all deaths in this age group) (112). Between 2000 and 2016, there were important improvements: a decrease in the incidence of the disease (from 2.0 to 1.75 per child year),

in the number of diarrhoeal deaths (from 1204 538 to 445 600), and in the global mortality rate (from 173.3 to 70.6 per 100.000). In 2016, the highest rates of under-five diarrhoeal deaths were mainly in the SSA, Figure 8.

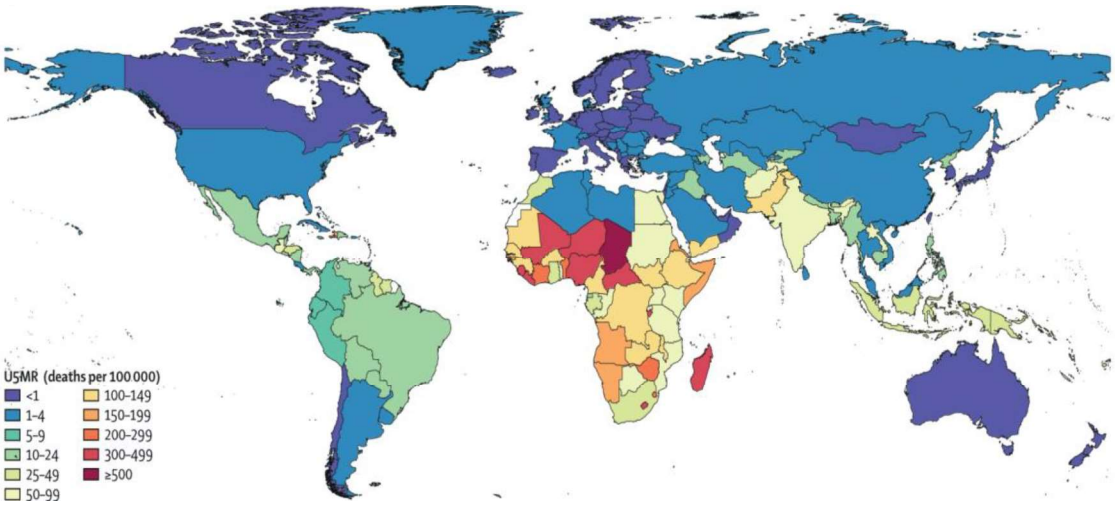

Figure 8. Diarrhoea mortality rate in children under-five years of age (2016)
Source: (112)

To address information related to the burden, factors, aetiology and risk factors of diarrhoea, a three-year prospective, age-stratified, matched controlled study of moderate-to-severe diarrhoea was conducted between December 2007 and March 2011 (10). The study, also known as Global Enteric Multicenter Study (GEMS), was conducted in seven sites located in Africa (Kenya, Mali, Mozambique, and Gambia) and Asia (Bangladesh, India, and Pakistan), and included 9.439 children with moderate-to-severe diarrhoea (cases), and 13.129 children without diarrhoea (control group) (10).

GEMS found that Rotavirus, *Cryptosporidium* spp. enterotoxigenic *Escherichia coli* producing heat-stable toxin (ST-ETEC), and *Shigella* were the major pathogens contributing to moderate-to-severe diarrhoea (10). Rotavirus had the highest moderate-to-severe diarrhoea attributable-fraction of any pathogen in all sites in infants (0-11 months) and in four sites in toddlers (12-23 months). The risk of dying during the follow-up was 8.5-fold higher in cases than in controls. The pathogens ST-ETEC and typical enteropathogenic *Escherichia coli* (in children 0-11 months), and *Cryptosporidium* spp. (12-23 months) were associated with increased risk of death (10). After enrolment, the

mean HAZ became significantly lower in children with moderate-to-severe diarrhoea than in controls, confirming the impact diarrhoeal disease in the nutritional status of children (10). *Giardia lamblia* was significantly more frequent in controls than in cases, which demonstrates that, even in the absence of diarrhoea, children are exposed to enteropathogens with important long-term consequences on their health and development (10, 115).

Another important study was a community birth cohort conducted between 2009 and 2014 in eight sites in Africa (South Africa, Tanzania), Asia (Bangladesh, India, Nepal, Pakistan) and South America (Brazil and Peru), also known as a MAL-ED: *The Etiology, Risk Factors, and Interactions of Enteric Infections and the Consequences for Child Health and Development Project* (116). MAL-ED birth cohort included 2145 children under two years and enteropathogens were detected in both diarrhoeal (76.9%) and non-diarrhoeal samples (64.9%) (116). In the first year of a child´s life, the study found that five pathogens, mainly norovirus GII (5.2%), rotavirus (4.8%), *Campylobacter* spp. (3.5%), astrovirus (2.7%) and *Cryptosporidium* spp. (2.0%) were the major pathogens associated with diarrhoea; while *Campylobacter* spp (7.9%), norovirus GII (5.4%), rotavirus (4.9%), astrovirus (4.2%) and *Shigella* (4.0%) were the most frequent in the second year of life (116). *Giardia lamblia* was not associated with diarrhoea in any site or age group (116).

Meanwhile, the stool samples from MAL-ED were reanalysed using molecular methods to reassess estimates of diarrhoea (117). According to the results, viral diarrhoea (36.4%) was more common than bacterial (25.0%) and parasitic diarrhoea (3.5%) (117), with only ten pathogens accounting for almost 96.0% of attributable episodes per 100 child-years, namely *Shigella* (26.1), sapovirus (22.8%), rotavirus (20.7%), adenovirus 40/41 (19.0%), enterotoxigenic *Escherichia coli* (18.8%), norovirus (15.4%), astrovirus (15%), *Campylobacter jejuni* or *C. coli* (12.1%), *Cryptosporidium* spp. (5.8%) and typical enteropathogenic *Escherichia coli* (5.4%) (117).

The effect of enteric infections on linear growth in children less than two years, based on a longitudinal analysis of MAL-ED, was recently reported by Rogawski and colleagues (118). The mean episodes per child caused by any enteropathogens was 2.1 during the first two years of a child´s life: viral (1.4), bacterial (0.9), and parasitic agents (0.1

episodes per child). Bacterial and parasitic diarrhoeal infections were more negatively associated with reduced linear growth after three months than viral agents (118). However, asymptomatic infections (*Escherichia coli*, *Giardia lamblia*, *Campylobacter*, *Shigella*, and *E. bieneusi*) were more commonly associated with reductions of LAZ than diarrhoeal infections in children with two years of age (118). High prevalences of asymptomatic infections were associated with a LAZ reduction of -0.41 (-0.61 to -0.21) compared with low prevalence of asymptomatic infections, enhancing the need to prevent asymptomatic or subclinical infections to improve stunting, Figure 9.

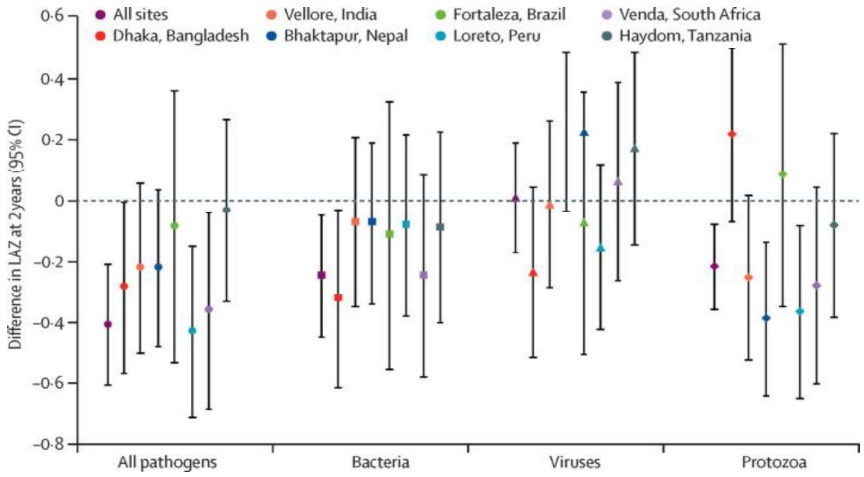

Figure 9. Site-specific effects of enteropathogen infections in non-diarrhoeal stools on height at age 2 years

Difference in LAZ at age 2 years between site-specific high and low combined pathogen prevalence in non-diarrhoeal stools among 1469 children in the MAL-ED. Bacteria (*Campylobacter*, *Shigella*, enteroaggregative *Escherichia coli*, typical enteropathogenic *E coli*, atypical enteropathogenic *E coli*, and enterotoxigenic *E coli*); viruses (norovirus, adenovirus 40/41, astrovirus, and sapovirus) and protozoa (*Giardia*, *Cryptosporidium*, and *Enterocytozoon bieneusi* - intracellular parasitic fungus). Estimates were adjusted for site, enrolment LAZ, sex, socioeconomic status, exclusive breastfeeding in the first 6 months, and maternal height. LAZ=length-for-age Z-scores. Source: (118)

1.3.2. Household food insecurity, inadequate care and feeding practices

Household food insecurity, inadequate care and feeding practices can influence the relation between nutrition and infection, and are identified as underlying causes of malnutrition, according to the UNICEF framework presented in Figure 6-A.

Household food insecurity

Food security exists *when all people, at all times, have physical and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for*

an active and healthy life (27). The prevalence of undernourishment (PoU) is an indicator that measures the probability that a randomly selected individual from the reference population is found to consume insufficient food to meet dietary energy required to maintain a normal, active and healthy food (119).

The PoU has been included in international nutrition goals. The WFS goal, established in 1996, aimed *to eradicate hunger in all countries, with an immediate view to reducing the number of undernourished people to half no later than 2015* (27). Additionally, the first Millennium Development Goal (MDG1) aimed to *reduce by 50% the proportion of undernourished people who suffer from hunger between 1990 and 2015* (29). At the present, the PoU is an indicator for monitoring the first target of SDG 2 (indicator 2.1.1, Figure 6-B) (36).

The PoU is presented in Table 7 considering three different periods (1990-1992, 2000-2002 and 2014-2016) and achievements towards WFS and MDG. As shown, in 1990-92, 18.6% of total population was undernourished, of which 98.0% lived in developing regions: 74.9% in Asia and 18.3% in Africa (119). Between 1990-92 and 2014-16, the PoU fell from 18.6% to 10.9%. This was a decline of 216 million people, while the world population increased by 2 billion. Despite the progress, undernourished people remained highly concentrated in developing regions (98.2%) and the WFS was not globally achieved (119).

Several factors could have contributed to these results, such as the increase of food prices, droughts, weak agricultural growth, and political instability (119). Wars are also an important factor, since people living in countries affected by conflict are usually more vulnerable to food insecure and malnutrition, although these factors can also be founded in peaceful countries (68).

In 2014-16, Africa presented the highest proportion of undernourished people (20.0%) (68, 119). However, substantial differences are noted across sub-regions (119): SSA is the region with the highest proportion of undernourished people (23.2%), while Northern Africa attained a prevalence below five (<5%). SSA is also the second region with the highest burden in absolute numbers, which have increased from 175.7 to 220 million people (from 1990-92 to 2014-16, respectively), Table 7.

Table 7. Prevalence of undernourishment and achievements towards World Food Security and Millennium Development Goal

Region	Prevalence of undernourishment (PoU)						Change*	
	1990-1992		2000-2002		2014-2016		Towards WFS Target[#]	Towards MDG target[¶]
Sub-region	No. millions	%	No. millions	%	No. millions	%	%	%
World	**1 010.6**	18.6	**929.6**	14.9	**794.6**	10.9	-21.4	-41.6
Developed regions	**20.0**	<5.0	**21.2**	<5.0	**14.7**	<5.0	-26.3	na
Developing regions	**990.7**	23.3	**908.4**	18.2	**779.9**	12.9	-21.3	-44.5
Africa	**181.7**	27.6	**210.2**	25.4	**232.5**	20.0	27.9	-27.7
Northern Africa	**6.0**	< 5.0	**6.6**	< 5.0	**4.3**	< 5.0	-27.9	na
Sub-Saharan Africa	**175.7**	33.2	**203.6**	30.0	**220.0**	23.2	25.2	-30.1
Eastern Africa	**103.9**	47.2	**121.6**	43.1	**124.2**	31.5	19.6	-33.2
Middle Africa	**24.2**	33.5	**42.4**	44.2	**58.9**	41.3	143.7	23.2
Angola	**6.9**	63.5	**7.0**	48.9	**3.2**	14.2	-52.1	-77.6
Southern Africa	**3.1**	7.2	**3.7**	7.1	**3.2**	5.2	2.3	-28.0
Western Africa	**44.6**	24.2	**35.9**	15.0	**33.7**	9.6	-24.5	-60.2
Asia	**741.9**	23.6	**636.5**	17.6	**511.7**	12.1	-31.0	-48.9
Caucasus and Central Asia	**9.6**	14.1	**10.9**	15.3	**5.8**	7.0	-39.9	-50.8
Eastern Asia	**295.4**	23.2	**221.7**	16.0	**145.1**	9.6	-50.9	-58.5
South-Eastern Asia	**137.5**	30.6	**117.6**	22.3	**60.5**	9.6	-56.0	-68.5
Southern Asia	**291.2**	23.9	**272.3**	18.5	**281.4**	15.7	-3.4	-34.4
Western Asia	**8.2**	6.4	**14.0**	8.6	**18.9**	8.4	129.5	32.2
Latin America and Caribbean	**66.1**	14.7	**60.4**	11.4	**34.3**	5.5	-48.0	-62.2
Caribbean	**8.1**	27.0	**8.2**	24.4	**7.5**	19.8	-7.2	-26.6
Latin America	**58.0**	13.9	**52.1**	10.5	**26.8**	<5.0	-53.3	na
Central America	**12.6**	10.7	**11.8**	8.3	**11.4**	6.6	-9.6	-38.2
South America	**45.4**	15.1	**40.3**	11.4	**ns**	<5.0	<-50.0	na
Oceania	**1.0**	**15.7**	**1.3**	16.5	**1.4**	14.2	51.5	-9.9

[*] Change calculated considering the period from 1990-92 to 2014-16. [#] Achieving the WFS target means reducing the number by more than half (<-50%); progress, but insufficient to achieve WFS target means reducing the number by less than half (>50%); or an increase in the number of people undernourished (>0.0%); [¶] Millennium Development Goal 1, target 1c: halve between 1990–92 and 2015, the proportion of people suffering from undernourishment, or reduce this proportion below 5 percent. *na* means not applicable. Source: (119)

In Asia, the proportion of undernourished people almost halve from 1990-92 (23.6%) to 2014-16 (12.1%), though with variations within sub-regions, with Southern Asia presenting a higher prevalence at the end of the period (15.7%) compared with the other Asian sub-regions, Table 7. Furthermore, Southern-Asia had the lowest reduction of undernourished people (-3.4%), compared with the remaining sub-regions, and is now the region with the highest burden of undernourishment in absolute numbers (281 million people, of which 194.6 million are in India) (119).

The slow progress and increase of undernourishment in Western Asia was a result of the rapid population growth in the region (119). The success achieved in South-Eastern and

Eastern Asia (including China) in both nutrition targets was possible due to overall economic growth that have contributed to the improved access to food in rural areas (119). Latin America have achieved both nutrition targets, but with differences among countries (119).

Between 1990-92 and 2014-16, Latin America and Caribbean was the region with the greatest reduction of undernourished people (from 66.1 to 5.5 million, -48%), especially in Latin America (from 58.0 to less than 5 million) and South America (from 45.4 to less than 5 million), In contrast, since 1990-92, Oceania have increased the number of undernourished people from 1.0 to 1.4 million in 2014-16, too slow to reach the WFS target.

Of the total of 129 countries monitored, 72 (55.8%) have reached the MDG 1c target, of which a list of 29 countries achieved also the WFS goal, where Angola is included (119). At the end of the MDG period, the global reduction of the proportion of undernourished people estimated was 41.6%, Table 7. Despite being slightly lower than the reduction rate required for MDG1 target, from a development perspective the target was considered to be achieved (119).

According to recent data, in 2016 the estimated number of undernourished increased to 815 million, which can be related to greater numbers of violent conflict, wars, displacement of populations, drop of economies (68). These figures suggest that achieving the nutrition goal of the Sustainable Agenda for 2030 will be challenging.

Inadequate care and feeding practices

Of the 17 SDGs, feeding practices are more directly related to SDG 2 - *End hunger, achieve food security and improved nutrition and promote sustainable agriculture;* and SDG 3 - *Ensure healthy lives and promote well-being for all at all ages* (36).

WHO and UNICEF recommend (120):

- Early initiation of breastfeeding (within the first hour of birth);
- Exclusive breastfeeding in children under 6 months (continue with exclusive breastfeeding with no other foods or liquids during the first six months of a child´s life, and providing counselling to mothers);

- Continued breastfeeding in children 6-24 months, with appropriate complementary solid food (continued breastfeeding at one year includes the proportion of children aged 12-15 months who are fed breastmilk, and at two years those with 20-23 months).

Globally, it is estimated that 44% of infants are breastfed within the first hour of birth, 40% aged 0-5 months are fed exclusively by breastmilk, and 60% of those aged 6-24 months are continued breastfed, mostly those at 1 year (74%), compared with at age of two (45%) (120). Breastfeeding rate varies considerably depending on the region of the world. The prevalence of all indicators (with the exception of early initiation) are higher in low- and middle-income countries compared with high-income countries (121). This trend is also observed within countries, with poorer individuals breastfeeding for longer periods compared with the richest ones (121, 122).

Considering the benefits of adequate breastfeeding practices, the 2030 agenda aim is to increase the global rate of early breastfeeding to 70%, exclusive breastfeeding to 60%, continued breastfeeding at age 1 to 80%, and continued breastfeeding at age 2 to 60% (120).

Adequate care of the caregivers is extremely important, since it requires time, persistence and dedication to ensure that the children are well fed in frequency, quality and quantity, which can influence the child's development and growth. Supporting women to breastfeeding is a social responsibility, including governments, legislators, policy makers and civil society (123). Despite this, there are other several factors that can contribute to the delayed onset of breastfeeding, such as health (mother or new-born llness), education (little information on the benefits of breastfeeding), industry (access to free samples of breastmilk substitutes) and cultural beliefs (121).

1.3.3. Unhealthy environment and inadequate health services

Unhealthy environment and inadequate health services are identified as underlying causes of the UNICEF conceptual framework (Figure 6-A).

Unhealthy environment

The access to safe water, good sanitation and hygiene conditions is essential to health of communities, and has a great impact on nutrition (98, 102, 124). WASH is a term usually

related to a group of interventions, such as handwashing with soap, water quality and quantity, sanitation, food hygiene and environmental hygiene (124). Progress of WASH is monitored through the indicators described in Figure 10. and access to safe drinking water, sanitation and hygiene is included in the SDGs (Goal 6) as shown in Table 8 (36, 125).

Drinking Water ladder	Sanitation ladder	Handwashing ladder
Safely managed Drinking water from an improved water source which is located on premises available when needed and free from faecal and priority contamination	**Safely managed** Use of an improved sanitation facility which is not shared with other households and where excreta are safely disposed in situ or transported and treated off-site	**Basic** Hand washing facility with soap and water in the household
Basic Drinking water from an improved source provided collection time is not more than 30 minutes for a roundtrip including queuing	**Basic** Use of improved facilities which are not shared with other households	
Limited Drinking water from an improved source where collection time exceeds 30 minutes for a roundtrip including queuing	**Limited** Use of improved facilities shared between two or more households	**Limited** Handwashing facility without soap or water
Unimproved Drinking water from an unprotected dug well or unprotected spring	**Unimproved** Use of pit latrines without a slab or platform, hanging latrines and bucket latrines	**No facility** No handwashing facility
Surface water Drinking water directly from a river, dam, lake, pond, stream, canal or irrigation channel	**Open defecation** Disposal of human faeces in fields, forest, bushes, open bodies of water, beaches or other open spaces or with solid waste	

Figure 10. Monitoring WASH for the SDGs

For water, improved sources include piped water, boreholes or tubewells, protrected dug wells, protrected springs and package or delivered water; For sanitation, improved facilities include flush/pour flush to piped sewer system, septic tank or pit latrine; ventilated improved pit latrine composting toilet or pit latrine with slab. Adapted from (126, 127)

Table 8. WASH and Sustainable Development Goals

WASH SECTOR GOAL	SDG GLOBAL TARGET		SDG GLOBAL INDICATOR	
Ending open defecation	6.2	By 2030, achieve access to adequate and equitable sanitation and hygiene for all and **end open defecation**, paying special attention to the needs of women and girls and those in vulnerable situations	6.2.1	Population practising **open defecation**
Achieving universal access to basic services	1.4	By 2030, ensure all men and women, in particular the poor and vulnerable, have equal rights to economic resources, as well as **access to basic services**...	1.4.1	Population living in households with access to basic services (including **basic drinking water, sanitation and hygiene**)
Progress towards safely managed services	6.1	By 2030, achieve universal and equitable access to **safe and affordable drinking water** for all	6.1.1	Population using **safely managed drinking water services**
	6.2	By 2030, achieve access to **adequate and equitable sanitation and hygiene** for all and end open defecation, paying special attention to the needs of women and girls and those in vulnerable situations	6.2.1	Population using **safely managed sanitation services**
			6.2.1	Population with a basic **handwashing facility** with soap and water available on premises

Source: (128)

Untreated excreta contaminate lagoons, lakes and rivers, some of them used as drinking water sources, for bathing, washing clothes and other household purposes (125, 129). Consequently, unsafe WASH can contribute to the faecal oral transmission of several pathogens, and influence the health of individuals, mainly children, through illnesses such as diarrhoeal diseases and parasitic infections. In 2012, unsafe WASH have caused more than 842.000 diarrhoea deaths (129).

Providing safe WASH is an opportunity to end cholera epidemics by 2030 and is important to respond to emergencies and minimize the risks of infection transmission (e.g., prevent the transmission of outbreaks such as Ebola Virus Disease). The SDG 3 (target 3.3) includes the reduction of neglected tropical diseases (NTDs) and water-borne diseases (36), including malaria and other NTDS such as schistosomiasis, filariasis, Chikungunya, Zika, Japanese encephalitis and West-Nile virus (129).

It is estimated that 71% of global population used a safely managed drinking water service in 2015. However, in SSA only 24% did it. In this region, 58% of people still use untreated surface water sources. Considering sanitation, in 2015, 39% of global population used a safely managed sanitation service. Open defecation is still being used for 10.892 million people in the world, mainly in SSA and southeast Asia. Open defecation practice has fell

between 2000 and 2015, except in SSA, where population growth has contributed to an increase from 204 to 220 million people (128).

Health care services

Achieve Universal Health Coverage (UHC), including financial risk protection, access to quality essential health-care services and access to safe, effective, quality and affordable essential medicines and vaccines for all is included in the SDG 3 (target 3.8) (36, 130). This target is assessed by two indicators: coverage of essential health services (3.8.1) and the proportion of a country's population with catastrophic spending on health, defined as large household expenditure on health (3.8.2) (131).

The UHC is based on the concept that all people receive the health service they need (health promotion, prevention, treatment, rehabilitation and palliative care) and with quality, to achieve potential health gains (130).

A specific index (UHC coverage index) was created for assessing progress of health coverage in countries during SDGs (131), which is based on indicators grouped into four categories: i) reproductive, maternal, new-born and child health; ii) infectious diseases; iii) noncommunicable diseases and; iv) service capacity and access and health security (131). The methodological aspects can be found in greater detail in the report Tracking Universal Health Coverage: 2017 Global Monitoring Report (131).

Table 9 presents the means of UHC service coverage index by regions. As it shows, the global value of UHC coverage index is 64, with values ranging from 22 to 86 among countries in 2015 (131). However, the coverage index differs across regions, with Eastern Asia and North America and Europe achieving all the value of 77, while the SSA registered the lowest value with 42 (131).

High-income countries tend to have high values on the index, while the lowest values are seen among low-income countries and some countries affected by armed-conflict. Indeed, there is a growing number of people who are spending at least 10 percent of their household budgets on out-of-pocket health expenses (130, 131). Thus, inequalities persist and many children still not accessing vital healthcare services, Table 9.

Table 9. Means of UHC service coverage index and its component indices by regions

Area	UHC service coverage index	RMNCH	Infectious diseases	NCDs	Service capacity and access
Global	**64**	**75**	**54**	**63**	**71**
Africa	**46**	**55**	**40**	**67**	**37**
Northern Africa	64	73	50	62	77
Sub-Saharan Africa	42	51	37	69	27
Asia	**64**	**75**	**51**	**63**	**71**
Eastern Asia	77	86	64	64	99
Southern Asia	53	66	41	64	47
South-Eastern Asia	59	78	45	59	63
Central Asia	70	81	56	58	93
Western Asia	65	69	59	57	79
Europe and Northern America	**77**	**88**	**73**	**58**	**96**
Latin America and the Caribbean	**75**	**81**	**65**	**68**	**88**
Oceania	**74**	**83**	**71**	**62**	**84**

NCDs: noncommunicable diseases; RMNCH: reproductive, maternal, newborn and child health; UHC: Universal Health Coverage. Adapted from: (131)

Improving the primary healthcare is the most cost-effective way to ensure access to essential health services along with good governance, supply of medicines, health technologies and well-functioning health information systems (130).

Challenges in the health systems differ across countries. In developing regions, in addition to infectious diseases, national health systems have to join efforts to face the "nutrition transition". This means that health care services in some countries will have to deal with the co-existence of underweight and overweight in the same households (5).

1.4. Strategies to improve nutritional status

The first series of Lancet Maternal and Child Nutrition 2013 presented a framework for actions to improve fetal and child nutrition, Figure 11. As shown, strategies are included based on three different groups: i) nutrition specific interventions; ii) nutrition sensitive interventions; and iii) strategies focused on the politics, resources and legislation at national and global levels (5). Some of these strategies are addressed to mothers, since the nutritional status during the pregnancy is important for fetal growth. However, in this thesis, only child-related strategies will be addressed.

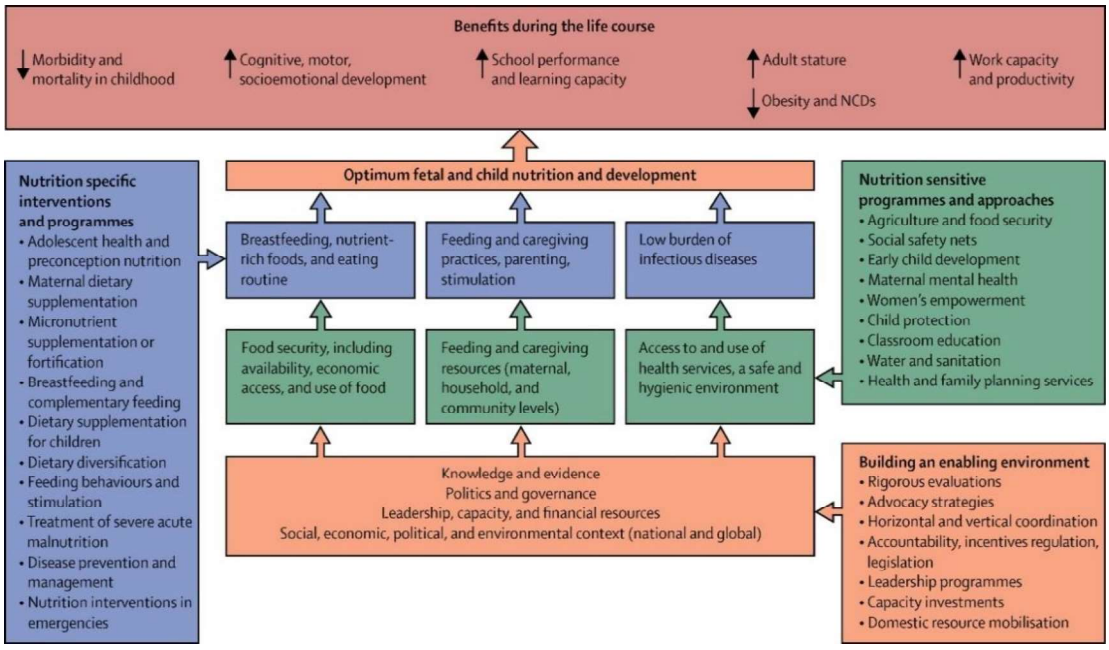

Figure 11. Framework for actions to improve fetal and child nutrition and development

Source: (5)

The most common interventions to improve the child´s health are the promotion of exclusive breastfeeding during the first six months of life; adequate complementary feeding in addition to breastfeeding in children between 6 and 23 months of age; improved WASH; immunization; deworming; and treatment of common diseases such as diarrhoea, pneumonia, malaria and intestinal parasites (9, 67, 132-134)

Breastfeeding

Breastfeeding has important short-term and long-term consequences for the child and the mother (121, 135, 136). A meta-analyses recently published in the *Breastfeeding Lancet Series*, described the impact of breastfeeding promotion on health, nutrition and developmental outcomes (121). Regarding mortality, the results have found that children who were breastfed had lower risk of death compared with those not breastfed, in both low-middle and high-income countries. There was also benefits of breastfeeding in reducing hospital admissions for diarrhoea (by 72%) and respiratory infections (by 57%) in low-middle income countries. Moreover, longer periods of breastfeeding were associated with a reduction by 26% of the odds of overweight or obesity, and with higher performance in intelligence tests in children (increase of 3.4 intelligence quotient points) (121).

WASH

Providing safe WASH can contribute to the interruption of the transmission of faecal pathogens and, thus, it is important for preventing diseases transmission (129), Figure 12.

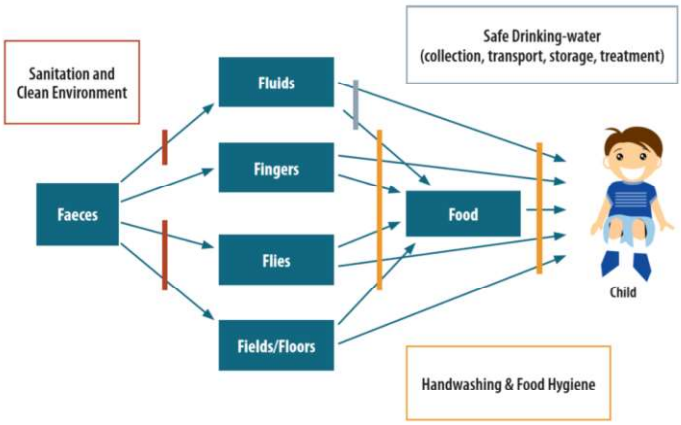

Figure 12. Interrupting transmission of pathogens providing adequate access to water, sanitation and hygiene
Source: (124)

However, research results showing the effect of WASH interventions on improved nutritional status have been inconsistent (124). A Cochrane systematic review published in 2013 concluded that available data from 14 cluster-randomized trials, with an intervention period ranging from 9 to 12 months, is suggestive of a small benefit of

WASH interventions (solar disinfection of water, provision of soap, and improvement of water quality) on growth of children under-five years of age (137). However, the short time of interventions and the fact that none of studies were of high quality methodology were pointed as limitations in the Cochrane review (137).

The effect of WASH and nutritional interventions on diarrhoea and child growth were assessed in a cluster-controlled trial conducted in a rural area of Kenya between 2012 and 2014 (138). After two-year of follow-up period, the study concluded that none of the interventions reduced diarrhoea and only intervention including nutrition counselling and nutrient supplementation improved growth (138). In contrast, a research conducted in four sites (Ethiopia, India, Peru, and Vietnam), found that access to improved sanitation was more frequently associated with reduced stunting risk than improved water (139)

Vitamin A supplementation

Vitamin A supplementation is a low-cost intervention recommended by WHO (44). A recent Cochrane systematic review published in 2017 have assessed the effect of Vitamin A supplementation for preventing morbidity and mortality in children 6 months to five years. Findings of this review are consistent with a reduction in morbidity and mortality in children, the later ranging from 12-24% of reduction (140). Given the benefits, supplementation continues to be recommended. However, the effects on growth have been less studied. Results from a randomised controlled trial assessing the effect of vitamin A supplementation on height and weight increments among preschool children in Indonesia, have showed greater benefits in children with very low serum retinol concentrations compared with children who did not receive supplementation (141).

Universal salt iodization

Universal salt iodization and iodine supplementation have been included in many countries where iodine deficiency was identified as a public health problem. These are safe, cost-effective and sustainable strategies to ensure adequate intake of iodine (142). In 2017, 86.0% of total population used salt with iodine (93)

Zinc supplementation

The effect of zinc supplementation for preventing morbidity and promoting growth was assessed in a Cochrane systematic review published in 2014 and including 80 trials

(205.401 children 6 months-12 years of age) (143). It was found a decrease in diarrhoea morbidity and a very small improvement in height (0.0 to 0.2 HAZ better, moderate-quality evidence) in children who received zinc supplementation compared to control groups. Zinc supplementation have also lead to increased vomiting (143).

Another Cochrane systematic review published in 2017 aimed to evaluate oral zinc supplementation for treating children with acute or persisting diarrhoea. The review included 33 trials (10.841 children aged one month to five years), most of them conducted in Asian countries. According to the results, zinc supplementation may reduce the average duration of diarrhoea by near half a day and probably reduce the number of children whose diarrhoea persists until day seven. In malnourished children the effect was apparently greater, decreasing the duration of diarrhoea by around a day (144). Thus, these findings enhanced the benefits of zinc supplementation in areas where the prevalence of malnutrition is high.

Rotavirus vaccine

Given the burden of diarrhoea, with rotavirus being the leading cause during infancy, especially in low- and middle-income countries, (10, 112, 113, 145), immunization is a key strategy to reduce the diarrhoeal morbidity and mortality, along with promotion and treatment packages (solutions of low-osmolarity to prevent dehydration along with breastfeeding and zinc supplementation) (114). WHO recommends the introduction of rotavirus vaccines in all national immunization programmes worldwide, especially in south and south-eastern Asia and SSA (146).

Four oral, live, attenuated rotavirus vaccines, Rotarix™ (derived from a single common strain of human rotavirus G1P[8]); RotaTeq™ (pentavalent rotavirus reassortant with human G1, G2, G3, G4 and P[8]) ; Rotavac™ (naturally occurring bovine-human reassortant neonatal G9P, also called 116E); and RotaSiil™ (bovine-human reassortant with human G1, G2, G3 and G4 bovine UK G6P[5] backbone) are available internationally and WHO prequalified (147). Rotarix™ and RotaTeq™ have been licensed for use since 2006, while Rotavac™ and RotaSiil™ received prequalification almost ten years after, in 2018. All four vaccines are considered highly effective in preventing severe gastrointestinal disease, although the lower effectiveness reported in low-income countries (50%-64%) compared with upper-middle and high-income

countries (85-98%) (148). In August 2018, 96 countries have introduced rotavirus vaccines (149).

Deworming

Deworming, or preventive chemotherapy, is a public health strategy against soil-transmitted helminths (STHs), which can be caused by *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale*. These infections have been associated to impaired growth, deficit cognitive development and reduced education attendance (150). In areas where the prevalence of STH is 20.0% or higher, WHO recommends deworming using annual or biannual single-dose of albendazole (ALB) or mebendazole for preschool- and school-age children in order to reduce the worm burden and achieve a coverage of 75% of children until 2020 (150). Albendazole and mebendazole are safe drugs, but the efficacy diverges within STH. It is well established the benefits of deworming on reducing the worm burden and, consequently, decreases morbidity. However, it does not breaks the cycle of transmission (150).

The health benefits and cost-effectiveness of deworming strategy are under discussion. Recently, systematic reviews have reported little or no benefits of deworming children living in endemic areas for STH at the community level (infected and non-infected), including growth and haemoglobin levels (151, 152). Similarly, no significant effect of routine deworming was found on weight gain and on mortality of 2 million PSAC study carried out in India (153). Conversely, analysis of more than 320.000 PSAC have reported benefits of deworming: those who received treatment were less likely to be stunted and anaemic, supporting the implementation of deworming (154). Results are controversial and it is clear that further research with new approaches is needed (155).

1.5. Angola – country profile

Angola is a sub-Saharan country bordered by the Atlantic Ocean (west), Namibia (south), the Democratic Republic of Congo (north), and Zambia (east). Portuguese is the official language of Angola, but there are more than 18 national languages (156).

1.5.1. Population

The country is divided into eighteen provinces that covers an area of 1.246,700 km^2. According to the last census, in 2014 Angola had a total population of 25.8 million inhabitants (157), of which 26.9% lived in the capital Luanda and 19.4% were under-five years of age, Table 10 (157). According to the World Bank, in 2017 the population increased to 29.8 million (158).

Table 10. Population of Angola in 2014 (by region, provinces, age and sex)

		N	(%)
	Angola	25 789 024	(100.0)
Region	Urban	16 153 987	(62.6)
	Rural	9 635 037	(37.4)
Provinces	Bengo	356 641	(1.4)
	Cuanza Norte	443 386	(1.7)
	Namibe	495 326	(1.9)
	Cuando Cubango	534 002	(2.1)
	Luanda Sul	537 587	(2.1)
	Zaire	594 428	(2.3)
	Cabinda	716 076	(2.8)
	Moxico	758 568	(2.9)
	Lunda Norte	862 566	(3.3)
	Malanje	986 363	(3.8)
	Cunene	990 087	(3.8)
	Bié	1 455 255	(5.6)
	Uíge	1 483 118	(5.8)
	Cuanza Sul	1 881 873	(7.3)
	Huambo	2 019 555	(7.8)
	Benguela	2 231 385	(8.7)
	Huíla	2 497 422	(9.7)
	Luanda	6 945 386	(26.9)
Age	Under-five	4 998 148	(19.4)
	5-14 years	7 198 348	(27.9)
	15-64 years	12 980 098	(50.3)
	≥ 65 years	612 430	(2.4)
Sex	Women	13 289 983	(51.5)
	Men	12 499 041	(48.5)

Adapted from (157)

1.5.2. The war

After 14 years of armed-conflict, Angola gained the independence from Portugal in 1975 (156). Then, nationalist groups engaged a protracted and severe civil war between 1975-2002. The war contributed to a massive destruction of the infrastructures, with several areas heavily mined, and caused numerous deaths (from fighting, diseases, starvation) and forced migration (156, 159-161). More than 1 million of Angolans died, 4.5 million were displaced to other regions of the country, and 450.000 fled the country (156). This led to a humanitarian crisis, with negative consequences on the economy, on the health systems and on the social welfare (159, 161). In fact, the armed-conflict period affected negatively an entire nation, although in an unevenly way (159). Children were the primary victims of the war through malnutrition and lacking of immunization, especially in fighting areas where the production and delivery of food, and the public health system were compromised (159). During the war, the city of Luanda was one of the safest places and, for that reason, attracted many people to escape the fighting zones, to search relatives and to look for better educational and economic conditions (162).

1.5.3. Health System and main indicators

Once the war was over, Angola begun an enormous challenge period of rebuilding the national economy and improving the living conditions of the individuals (159). There was an intention of the government to implement a national decentralization in order to transfer the decision-making from the central level to the provincial and district level (156, 163).

Several problems have been reported in the Angolan health system, such as the lack of an effective administrative-financial system, insufficient number and inefficient distribution of health units, and poor quality of services (lack of equipment, drugs, electricity and water, poorly trained health professionals and lack of supervision in care) (156). Indeed, the health service coverage index registered for Angola is 36, lower than 44 for the WHO African region (and 64 for the global average value) (131). Meanwhile, Angola has made a strong investment in the collection of health information, mainly since 2005, such as the elaboration of provincial health maps and health surveys (156).

The health politics of the country are based on the long-term strategy Angola 2025: *join efforts to reduce poverty, improve the health status of the population, especially the most vulnerable groups to ensure greater longevity* (41). The main objectives included are:

- ✓ Combat diseases (including those of origin transmissible and parasitic diseases, and chronic diseases);
- ✓ Protect maternal health and support reproductive health;
- ✓ Reduce under-five mortality;
- ✓ Develop the primary health care network;
- ✓ Expand the secondary health care as second priority (hospitals);
- ✓ Consolidate the tertiary health care (differentiated health care);
- ✓ Increase the human resources (in quantity and quality);
- ✓ Develop a financial model with different participants (public sector, private sector and international aid).

Progress of the Human Development Index (HDI)[10] made in the after-war period translates important improvements achieved across the years, mainly in expected years of schooling and Gross National income per capita, which more than duplicated between 2000 and 2017. A great increase in the life expectancy at birth also occurred, from 47.1 to 61.8 between the same period, Table 11 (164).

Table 11. Progress of Human Development Index and its components in Angola

Dimension	**Indicator**	**2000**	**2010**	**2015**	**2017**
	HDI	0.387	0.520	0.572	0.581
A long and healthy life	Life expectancy at birth (years)	47.1	58.2	61.2	61.8
Access to knowledge	Expected years of schooling	5.1	8.6	11.0	11.8
	Mean years of schooling	4.4	4.7	5.0	5.1
Standard of living	Gross National income (GNI) per capita	2.443	5.421	6.251	5.790

Adapted from: (164)

[10] The Human Development Index (HDI) is a composite index measuring average achievement in three basic dimensions - a long and healthy life, access to knowledge and a decent standard of living.

According to the most recent statistical update of the Human Development Indices and Indicators, Angola is classified in the medium human development category with a HDI value of 0.581 (147 rank in a total of 189 countries and territories), Table 11 (164). Angola´s HDI value is above the average for SSA (0.537), but below the average for the group of countries with the same classification (0.645) (165). The great economic expansion observed in the country was mainly due to oil exportation (156). However, despite the macroeconomic growth achieved, the same level of progress was lacking in the health and social sectors (166).

The main indicators of Angola are described in Table 12. These indicators are related to poverty, health, nutrition, education and environment. All of them are essential to understand the living conditions and the major challenges of the country.

The Multidimensional Poverty Index (MPI) is an international measure of acute poverty based on 10 indicators of severe deprivations related to education, health and living standards, described in detail in Table 13 (167). Each dimension (and each indicator within the dimension) is equally weighted, and by applying the Alkire and Foster´s methodology described elsewhere (167), it results in a score ranging from 0 to 1.

The most recent Global MPI was published in 2018 and includes 105 countries, covering 77% of the global population. Angola is one of the countries included and analysis was based on data from the Demographic Health Survey conducted in 2015-2016 (MICS 2015-2016) (168).

The country obtained a multidimensional poverty score of 0.283, Table 12. According to the results, 51.2% of the Angolan population is multidimensionally poor, which means that they are deprived in more than one third (33.3%) of the weighted indicators described in Table 13. Moreover, the percentage of severe poverty (proportion of individuals deprived in 50-100% of the weighted indicators) is higher is rural areas (66.5%) compared with urban areas (12.9%), Table 12.

Table 12. Indicators of Angola related to poverty, health, nutrition, education and environment

	Indicator	Total	Urban (63.5%)	Rural (36.5%)	Year
Poverty	**Multidimensional poverty index** (0-1)	**0.283**	0.145	0.523	2018[a]
	Vulnerable to poverty (20%<deprivation<33.3%)	**15.5**	19.8	7.9	2018[a]
	Incidence of poverty (deprivation ≥ 33.3%)	**51.2**	29.9	88.2	2018[a]
	Severe poverty (50% < deprivation < 100.0%)	**32.5**	12.9	66.5	2018[a]
	Population below poverty line (PPP $1.90 a day)	**30.5**			2006-17[b]
Health	**Infant mortality rate** (per 1000 live births)	**44**	43	61	2016[c]
	Under-five mortality rate (per 1000 live births)	**68**	68	65	2016[c]
	Under-five mortality rate (per 1000 live births)	**81.1**	-	-	2017[d]
	Maternal mortality rate (deaths per 100,000 live births)	**477**	-	-	2015[e]
	Total fertility ratio (births per women)	**6.2**	5.3	8.2	2016[c]
	Birth attendance by skilled personnel	**50**	68	21	2016[c]
	Births delivered in a health facility (%)	**46**	65	17	2016[c]
	Physicians density	**0.17**			2009[f]
	Nurses density	**1.442**			2009[f]
	Malaria prevalence by RDT (6-59 months, %)	**13.5**	7.5	21.8	2016[c]
	Total HIV prevalence	**2.0**	2.1	1.5	2016[c]
Nutrition	**Stunting** U5 (%)	**38**	32	46	2016[c]
	Wasting U5 (%)	**4.9**	4.6	5.3	2016[c]
	Underweight U5 (%)	**19.0**	15.0	24.7	2016[c]
	Prevalence of undernourishment (total population)	**14.0**			2014-16[g]
	Overweight U5 (%)	**3.2**	3.8	2.5	2016[c]
	Exclusive breastfeeding (<6months, %)	**37.4**	36.8	38.2	2016[c]
	Complementary feeding (6-23 monthts, %)	**80.0**	78.4	82.4	2016[c]
	Minimum acceptable diet (6-23 months, %)	**13.3**	15.8	9.4	2016[c]
	Vitamin A supplementation (6-59 months, %)	**5.7**	7.3	3.3	2016[c]
	Consumption of iodized salt (households, %**)**	**88.5**	94.3	78.4	2016[c]
	Prevalence of anaemia in children (6-59 months, %)				
	- **Mild** (10.0-10.9 g/dl)	**30.7**	31.1	30.0	2016[c]
	- **Moderate** (7.0-9.9 g/dl)	**31.8**	31.2	32.7	2016[c]
	- **Severe** (<7.0g/dl)	**2.2**	2.1	2.4	2016[c]
	Prevalence of anaemia among women (15-49 years, %)	**47.7**			2014-16[g]
Education	**Literacy rate adult** (% ages +15)	**65.6**	79.4	41.1	2014[h]
	Expected years of schooling	**11.8**			2017[b]
	Mean years of schooling	**5.1**			2017[b]
Environment	**Drinking water estimates**				
	- At least basic	**41**	63	23	2015[i]
	- Limited (>30mins)	**16**	19	13	2015[i]
	- Unimproved	**19**	15	22	2015[i]
	- Surface water	**24**	3	42	2015[i]
	Sanitation estimates				
	- At least basic	**39**	62	21	2015[i]
	- Limited (shared)	**15**	27	5	2015[i]
	- Unimproved	**13**	7	17	2015[i]
	- Open defecation	**33**	3	56	2015[i]
	Hygiene estimates				
	- Basic	**25**	37	15	2015[i]
	- Limited (without water or soap)	**12**	13	12	2015[i]
	- No facility	**63**	50	73	2015[i]

[a]Source (168); [b]Source (164); [c]Source (40); [d]Source (169); [e]Source (170); [f]Source (171); [g]Source (172); [h]Source (157); [i]Source (128)
RDT: rapid diagnostic test. HIV: human immunodeficiency virus

Table 13. Multidimensional Poverty Index 2018 (dimensions, indicators and weights)

Dimension of poverty	Indicator	SDG	Deprived if living the household where	Weight
Health	Nutrition	SDG 2	An adult under 70 years of age or a child is undernourished	1/6
	Child mortality	SDG 3	Any child has died in the family in the five-period preceding the survey	1/6
Education	Years of schooling	SDG 4	No household member aged 10 years or older has completed six years of schooling	1/6
	School attendance	SDG 4	Any school-aged child is not attending school up to the age at which he or she would complete class 8	1/6
Standard of living	Cooking fuel	SDG 7	The household cooks with dung, wood, charcoal or coal	1/18
	Sanitation	SDG 11	The household´s sanitation facility is not improved (according to SDG guidelines) or it is improved but shared with other households	1/18
	Drinking water	SDG 6	The household does not have access to improved drinking water (according to SDG guidelines) or safe drinking water is at least a 30-minute walk from home, round trip	1/18
	Electricity	SDG 7	The household has no electricity	1/18
	Housing	SDG 11	Housing materials for at least one of roof, walls and floor are inadequate: the floor is of natural materials and/or the roof and/or walls are of natural rudimentary materials	1/18
	Assets	SDG 1	The household does not own more than one of these assets: radio, TV, telephone, computer, animal cart, bicycle, motorbike or refrigerator, and does not own a car or truck	1/18

Adapted from (173)

Currently, of the three dimensions considered to elaborate the index, in Angola the *standards of living* are the major contributor of deprivation in overall poverty (46.8%), followed by *education* (32.0%) and *health* (21.2%), Figure 13.

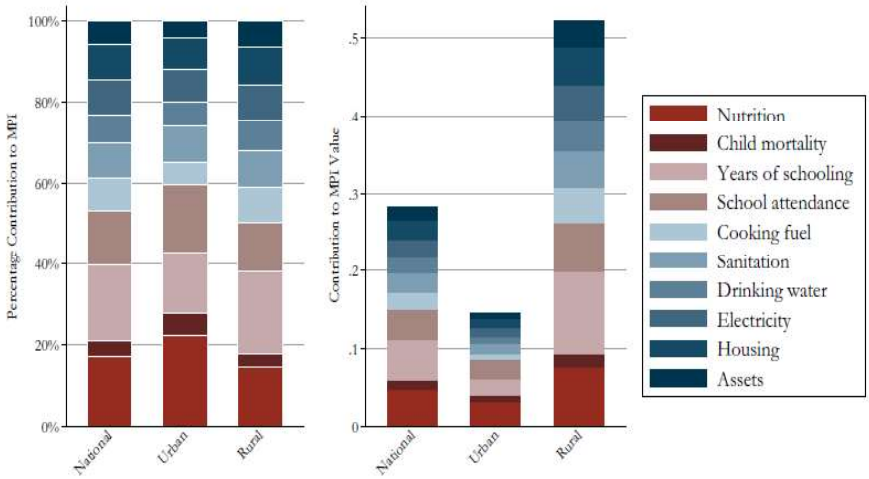

Figure 13. Indicator contributing to overall poverty by area, in Angola

Source: (168)

Among the 10 indicators, the largest contributors to poverty in Angola are deprivations in years of schooling (18.5%) and nutrition (17.3%), as shown in Figure 14. Considering the MPI by age group, the highest score is among children less than nine years (0.343) compared with older individuals. In this age group (0-9 years), nutrition is the indicator that most contributes to overall poverty with 19.8% (167).

Undernutrition has improved since the nineties, though it remains a public health problem. As Table 14 presents, during the first MICS (1996-1997), the prevalence of stunting in under-five Angolan children was 53.1% and it decreased across the years until 2007 achieving a percentage of 29.2%. However, in 2015-2016, the prevalence of stunting increased to 37.6%, which could be related with economic crisis faced in 2014 (174).

As shown in Table 14, wasting has maintained stable until 2002 (6.3%), increased to 8.2% in 2007, and decreased to 4.9% in 2016. Regarding underweight, it decreased progressively in the first decade (41.6% in 1996-1997; 30.5% in 2001-2002; and 15.6% in 2007). However, in 2015-2016, it has increased to 19.0%.

Table 14. Progress of stunting, wasting and underweight in Angola and targets to achieve by 2021

	1996-1997[a]	2001-2002[b]	2007[c]	2015-2016[d]	Target 2021[e]
	(%)	(%)	(%)	(%)	(%)
Stunting (HAZ<-2), %	53.1	45.2	29.2	37.6	< 5
Wasting (WHZ<-2), %	6.4	6.3	8.2	4.9	<5
Underweight (WAZ<-2), %	41.6	30.5	15.6	19.0	<10

[a] Source: (175); [b] Source:(176); [c] Source:(177); [d] Source:(40); [e] Source: (41)

In 2012, through the National Plan for Health Development 2012-2025 (41), Angola has made a commitment to achieve nutrition targets until 2021, including:

- ✓ Reducing chronic malnutrition in under-five children to less than 5%
- ✓ Reducing acute malnutrition in under-five children to less than 5%
- ✓ Reducing underweight in under-five children to less than 10%

Between 1990 and 2010, Angola achieved one of the fastest average annual reduction rate of stunting with 6.6% (after Saudi Arabia with 7.3% and before China and Brazil with 6.0% and 5.7%, respectively) (178). However, as presented in Figure 14, stunting reduction decelerated considerably, and adjusted projections considering the number of children in 2016 (1.82 million) indicate that national (2021) and international nutrition targets (2025) will remain to be achieved.

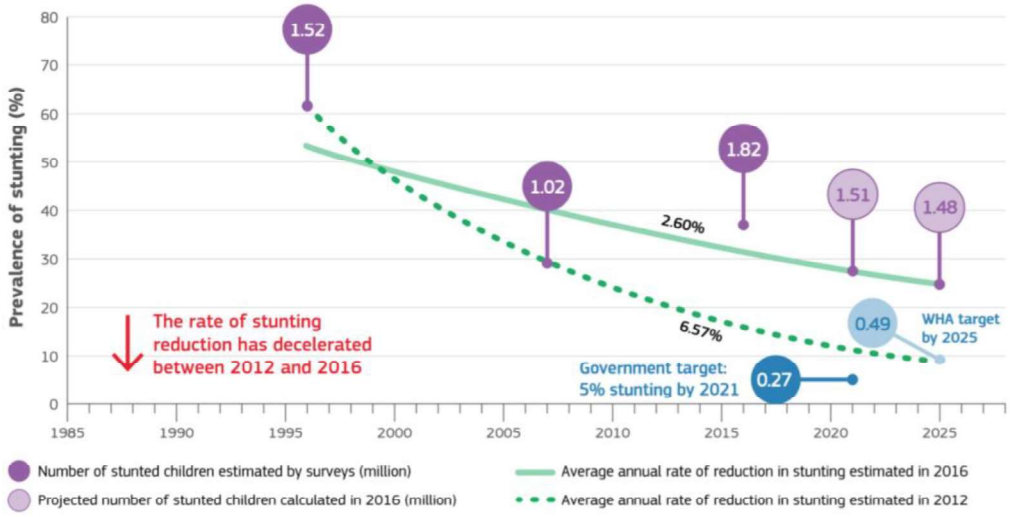

Figure 14. Progress of stunting prevalence and average annual rate of reduction
Source: (179)

It is important to understand the distribution of malnutrition within the country. Malnutrition differs across areas, with a higher proportion of children suffering from undernutrition in rural areas than urban areas, whereas overweight is more prevalent in urban areas, as showed previously in Table 12. Indeed, there are considerable differences in several indicators comparing rural to urban areas, which can influence the distribution of malnutrition: access to at least basic drinking water (23% vs 63%); open defecation practices (3% vs 56%); basic hygiene conditions (15% vs 37%); literacy rate adult (41.1% vs 79.4%); total fertility ratio (8.2% vs 5.3%); birth attendance by skilled personnel (21% vs 68%); and malaria (21.8 vs 7.5).

All these factors seem to contribute to a higher proportion of people living in a situation of severe poverty in rural areas compared with urban areas (66.5% vs 12.9%), Table 12

The national prevalence of exclusive breastfeeding in 2015/2016 was 37.4%, and the Angolan Government has the target of increasing exclusive breastfeeding to 85% until 2021 (180). Breastfeeding is more frequent in rural than urban areas (38.2% vs 36.8%, Table 12).

Differences of stunting prevalence are presented in Figure 15 for two different periods: 2006-07 and 2015-16 (39).

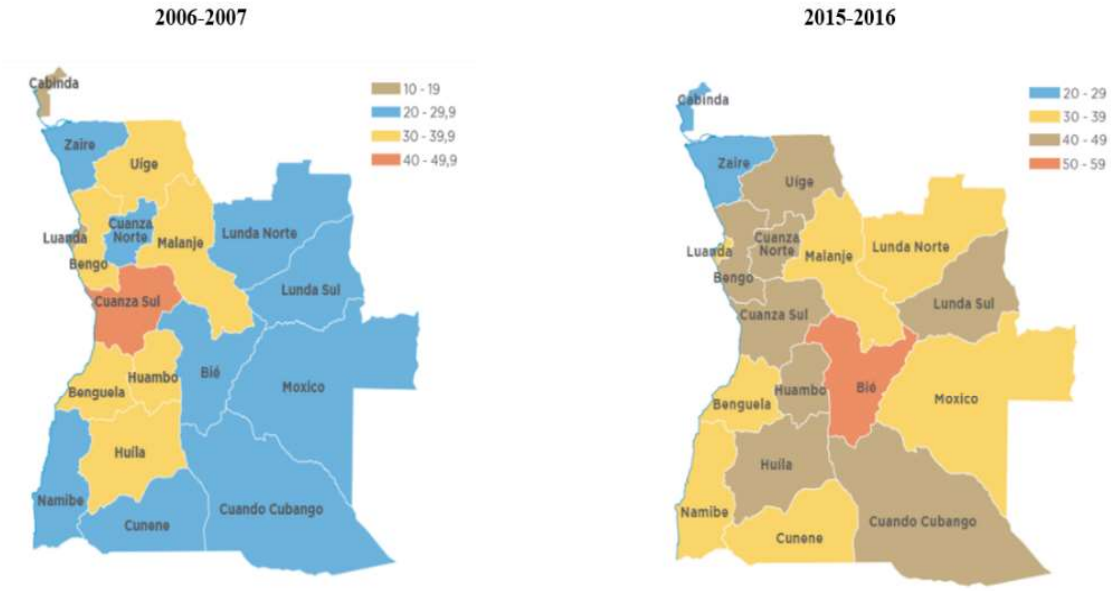

Figure 15. Prevalence of stunting in Angola in 2006-2007 and 2015-2016

Source: (174)

According to MICS 2015-2016, the provinces of Bié, Cuanza Sul and Cuanza Norte are in the three leading positions of moderate to severe stunting (50.8%, 48.8% and 44.5%, respectively), while Luanda, Zaire and Cabinda are those with the lowest prevalence registered (29.7%, 24.9% and 21.6%, respectively). The overall mean HAZ of under-five children was -1.5 Z-score, ranging from -2.0 to -1.0 Z-score across provinces, Table 15.

Regarding wasting, Cunene, Malanje and Huambo are the provinces with the highest prevalence levels (10.5%, 7.6%, and 6%, respectively); while Luanda, Cuanza Sul and Zaire registered the lowest prevalence of moderate-to-severe wasting (3.9%, 3.3% and 3.2%, respectively). The overall mean WHZ was -0.1 Z-score, ranging from -0.3 to 0.1 Z-score, Table 15.

Table 15. Stunting, wasting and underweight by province in Angola (2015-2016)

	STUNTING				WASTING				UNDERWEIGHT			
	Rank position (HAZ<-2)	**Moderate and severe (%)** (HAZ<-2)	**Severe (%)** (HAZ<-3)	**Z-score** (mean)	**Rank position** (WHZ<-2)	**Moderate and severe (%)** (WHZ<-2)	**Severe (%)** (WHZ<-3)	**Z-score** (mean)	**Rank position** (WAZ<-2)	**Moderate and severe (%)** WAZ<-2	**Severe (%)** WAZ<-3	**Z-score** (mean)
Angola	n.a	**37.6**	**15.2**	**-1.5**	n.a	**4.9**	**1.0**	**-0.1**	n.a	**19.0**	**5.6**	**-1.0**
Cabinda	18	21.6	5.8	-1.0	5	5.3	1.2	-0.2	18	10.4	2.0	-0.7
Zaire	17	24.9	7.3	-1.1	14	3.2	0.8	-0.2	17	12.2	4.6	-0.8
Uíge	8	41.7	16.9	-1.6	7	4.7	0.8	-0.3	8	21.5	6.1	-1.1
Luanda	16	29.7	10.4	-1.3	12	3.9	0.4	0.0	16	12.9	3.6	-0.7
Cuanza Norte	**3**	44.5	22.0	-1.6	11	4.0	1.2	0.0	7	21.6	4.0	-1.0
Cuanza Sul	**2**	48.8	22.8	-2.0	13	3.3	0.5	0.0	4	23.1	6.1	-1.2
Malanje	15	31.9	10.2	-1.3	**2**	7.6	2.5	-0.3	11	18.9	8.4	-1.1
Lunda Norte	11	38.7	19.9	-1.5	4	5.8	2.2	0.0	10	19.4	5.4	-0.9
Benguela	14	33.1	11.4	-1.4	8	4.6	0.5	-0.2	15	15.7	2.9	-0.9
Huambo	5	43.6	17.1	-1.7	**3**	6.0	2.0	-0.1	9	21.2	5.5	-1.1
Bié	**1**	50.8	19.5	-1.9	6	4.9	1.8	-0.1	6	21.7	7.5	-1.2
Moxico	12	38.5	17.3	-1.7	10	4.3	1.6	-0.1	5	21.8	7.0	-1.0
Cuando Cubango	6	42.9	20.5	-1.6	5	5.3	0.2	-0.2	3	23.9	7.5	-1.0
Namibe	13	33.8	15.9	-1.5	9	4.5	0.6	0.1	14	15.8	5.7	-0.8
Huila	4	43.8	21.7	-1.8	8	4.6	0.5	-0.3	2	27.8	9.8	-1.3
Cunene	10	39.3	17.0	-1.7	**1**	10.5	3.6	-0.6	1	30.8	9.3	-1.4
Luanda Sul	7	42.1	15.4	-1.6	10	4.3	1.6	0.1	13	17.1	4.8	-1.0
Bengo	9	39.7	12.1	-1.5	7	4.7	1.3	-0.1	12	17.2	3.7	-1.0

Provinces with the equal percentage (%) within the same category of undernutrition are ranked in the same position. n.a = not applied. Adapted from (40)

National data indicates an infant mortality rate (probability of dying between birth and age of 1 year per 1000 live births) of 44 per 1000 live births, and when considering children under-five years it increases to 68.0 deaths per 1,000 live births (40), as shown previously in Table 12. However, the international data estimates a higher under-five mortality rate than the national data: 81.1 deaths per 1,000 live births, the equivalent to 1 child in 12 dying before completing his or her fifth anniversary.

Infectious diseases have been a serious public health problem in children under-five years for many years in Angola. According to data extracted from Global Burden Disease (Institute for Health Metrics and Evaluation), enteric infections were the major cause of death in this age group in 1990. Though there has been a slight improvement, enteric infections remain among the three leading causes of death of under-five children, after maternal and neonatal factors, and before respiratory infections, as shown in Figure 16.

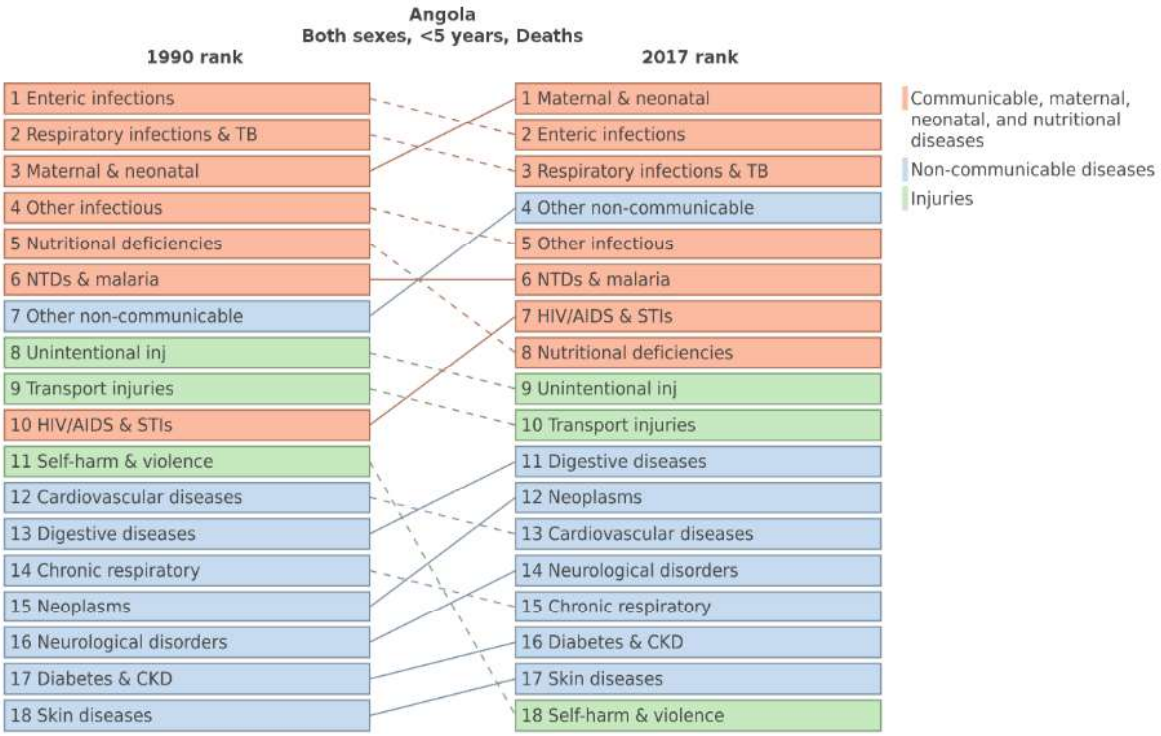

Figure 16. Cause of deaths in children under-five years in Angola in 1990 and 2017.
Source: (181)

Adequate nutrition is recognised as the key to sustainable social and economic development of the country, and the *Prevention and treatment of nutrition diseases* are

goals also included in the National Health Development Plan 2012-2025. Thus, several strategies are identified at national-level, and recognized as essential to reducing morbidity and mortality caused by malnutrition in children under five years (41).

- Inclusion of nutrition services in primary health care as an absolute priority;
- Reinforcement of micronutrient distribution and deworming with albendazole in children under five years of age;
- Establishment of a disease surveillance system due to food institutional and community-based micronutrients;
- Strengthening epidemiological surveillance of malnutrition;
- Promotion of breastfeeding soon after birth, exclusive up to 6 months and appropriate feeding practices after 6 months of age;
- Promotion of healthy eating habits and lifestyles;
- Iron fortification of staple food for the general population;
- Capacity-building efforts and nutrition-education and training opportunities;
- Strengthening community participation and family empowerment through skills family members;
- Mobilizing strategic partnerships for a multisectoral response.

Considering results from MICS 2015-16, only 13% of children aged 12-23 months and 9% of children 24-35 months received all-age appropriate vaccines, and it was reported that coverage increased with mother´s level of education (40). Of those who reported diarrhoea in the previous two weeks, 53% received oral-rehydration treatment, though almost 30% did not receive any treatment.

The strategy of deworming is also included in the NTD national program through the distribution of albendazole to school-age children. Soil-transmitted helminths are endemic and considered a public health problem in Angola (42, 43).

According to MICS 2015-16, 48% of infants was breastfed within the first hour. The median duration in months of exclusive breastfeeding was 3.1 months, and complementary breastfeeding was 18.7 months (40). Exclusive breastfeeding in the first 6 months was reported for 38% of children.

Considering other strategies such as the use of iodized salt, the survey registered a household coverage higher in urban than rural areas (95% vs 80%). Consumption of vitamin A supplement was reported in only 6% of under-five children, though 75% of those aged 6-23 months ate foods rich in this micronutrient in the 24 hours before the study. Moreover, only 6% (6-59 months) have received iron supplementation in the week before (40).

In 2013, Angola was the country with the highest mortality rate due to rotavirus (240 per 100,000 children under-five years of age) (182). To reduce severe diarrhoeal disease, Angola requested assistance from The Global Alliance for Vaccines and Immunizations (GAVI) to introduce Rotarix vaccine in the national immunization program in 2014. The existing data on child malnutrition and the enteric infections in Angola are scarce and are mainly transversal studies and focused on intestinal parasites infections (43, 183-185).

As will be explained further, in the Methodology section, this thesis intends not only to contribute with data on the etiological agents of diarrhoea (Results section – papers I, II and III), as well as to investigate longitudinally different treatment strategies of intestinal parasites on nutritional status of children (paper IV).

1.6. Objectives of this thesis

This thesis aims to:

1) identify the most frequent pathogenic agents (viral, bacteria and parasites) of diarrhoea and associated factors in children under-five years of age attending the referral hospital of Bengo province between September 2012 and December 2013;

2) provide baseline information on the detection rate, associated risks and molecular characterization of rotavirus infection, before the vaccine introduction in Angola, in children under-five years of age attending the referral hospital of Bengo province between September 2012 and December 2013

3) provide a molecular characterization of *Giardia lamblia* infection in children under-five years of age attending the Bengo General Hospital between September 2012 and December 2013

4) investigate if treatment of intestinal parasites (with or without previous diagnosis) in two different levels (individual or household) impacts on nutritional status of children between 2 and 5 years old, after a two-year follow-up period in Bengo province, Angola.

The general and specific objectives of this thesis and the corresponding articles are described in detail in Table 16. Furthermore, a conceptual framework, including questions addressed through these research studies, is presented in Figure 17.

Table 16. General and specific objectives of the thesis

Topic	General objectives	Specific objectives	Paper	Title
Malnutrition and diarrhoeal disease	1. To identify the most frequent **pathogenic agents of diarrhoea** in children under-five years of age attending the Bengo General Hospital from September 2012 and December 2013	a. To quantify the occurrence of undernutrition (stunting, wasting and underweight) and level of malnutrition (mild, moderate and severe) in children with diarrhoea; b. To quantify the occurrence of **virus** (rotavirus and adenovirus), **intestinal protozoa** (mainly *Giardia lamblia*, *Entamoeba histolytica/dispar/moskovskii*, *Cryptosporidium* spp.) and **intestinal helminths** (*Ascaris lumbricoides*, *Trichuris trichiura*, ancylostomidae, *Strongyloides stercoralis* and *Hymenolepis nana*) and **bacteria** (*Shigella* spp., *Salmonella* spp., *Campylobacter* spp. and *Escherichia coli* (EPEC, ETEC, EAEC, VTEC, EHEC and EIEC)) in diarrhoeal stool of children included; c. To quantify the occurrence of malaria in children included; d. To identify **risk factors** associated with the infections (age, sex, child malnutrition, symptoms, type of admission, exclusive and complementary breastfeeding, parents´ education, drinking water source and sanitation conditions)	**I**	***Etiology of diarrhoea in children younger than 5 years attending the Bengo General Hospital in Angola***
	2. To provide baseline information on **rotavirus** infection in children under-five years of age attending the Bengo General Hospital between September 2012 and December 2013 and **before the introduction of rotavirus vaccine** into the national vaccination schedule	e. To identify the occurrence of rotavirus and the most frequent genotypes in diarrhoeal stool of children included f. To explore associations between rotavirus infection and the sociodemographic characteristics and living conditions, nutritional status, breastfeeding, clinical features and seasonality	**II**	***Characterization of rotavirus infection in children with acute gastroenteritis in Bengo province, Northwestern Angola, prior to vaccine introduction***

Topic	General objectives	Specific objectives	Paper	Title
	3. To provide a molecular characterization of ***Giardia lamblia*** infection in children under-five years of age attending the Bengo General Hospital between September 2012 and December 2013	a. To identify the occurrence of *Giardia lamblia* in diarrhoeal stool of children included b. To perform a molecular characterization of *Giardia lamblia* using polymerase chain reaction (PCR) and DNA sequencing in the diarrhoeal stool of children included	**III**	***Molecular characterization of Giardia lamblia in children less than 5 years of age attending the Bengo General Hospital, Angola***
Interventions to reduce malnutrition (deworming and growth)	4. To investigate if treatment of intestinal parasites (with or without previous diagnosis) in two different levels (individual or household) impacts on nutritional status of children between 2 and 5 years old, after a two-year follow-up period.	c. To describe the sociodemographic characteristics and living conditions of the total children included and per group of intervention d. To quantify the occurrence of undernutrition (stunting, wasting and underweight) and level (mild, moderate and severe) at baseline and 4, 8, 12, 20 and 24 months of follow-up period e. To describe the effect of four different interventions in the growth of children after: • Giving **albendazole** every four months (without any previous knowledge of the infectious status) at children included (individual level); • Giving **albendazole** every four months (without any previous knowledge of the infectious status) at children included and their household members (household level); • **Screening for pathogenic intestinal parasites** every four months and providing **treatment** of positive cases to children included (individual level) • **Screening for pathogenic intestinal parasites** every four months and providing treatment of positive cases to children included and their household members (household level)	*IV*	***Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional status in Bengo, Angola: a four-arm randomised parallel trial***

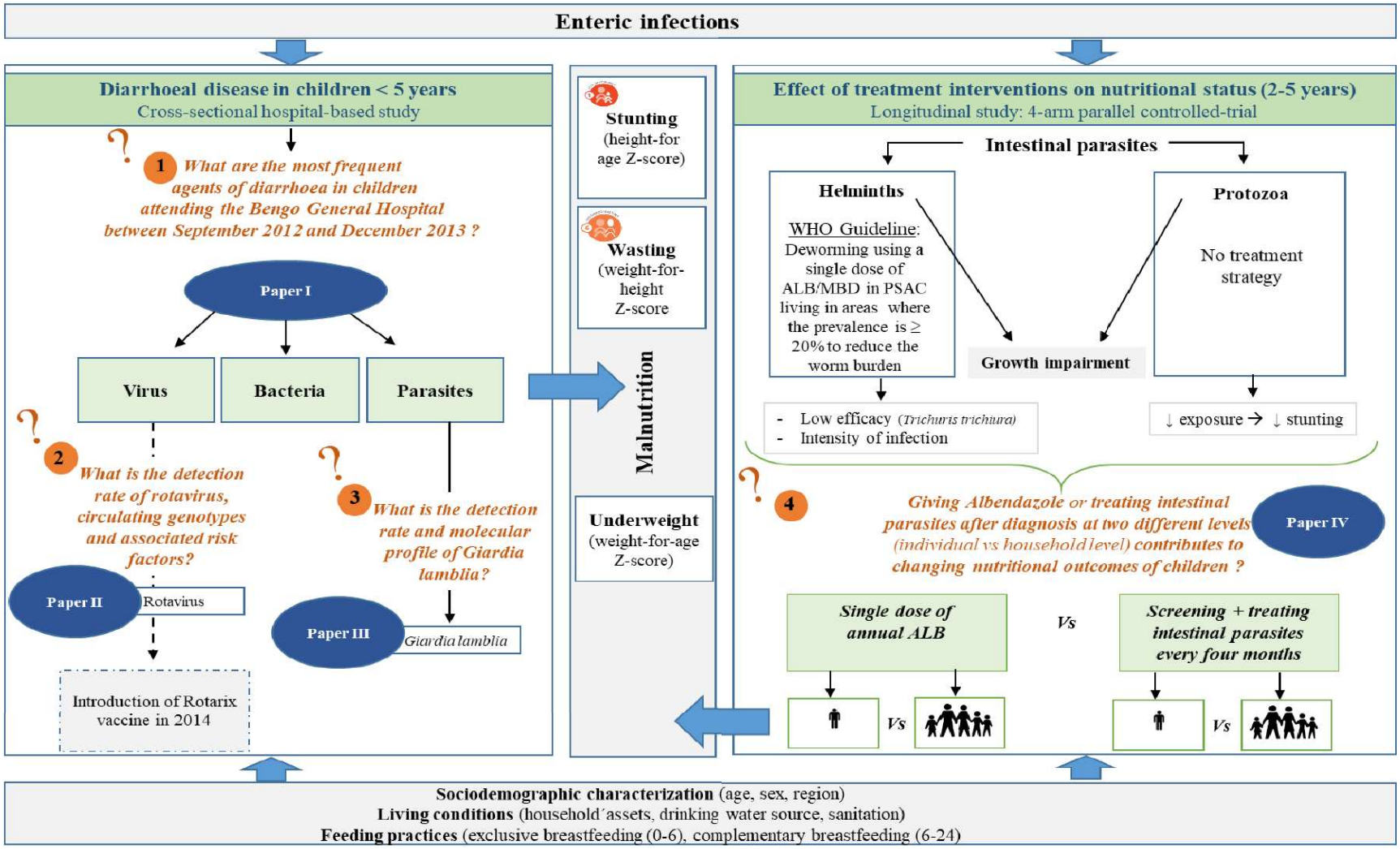

Figure 17. Research questions addressed in the thesis

1.7. Methodology

To answer the four research questions previously described in Figure 17, two main studies were performed: a cross-sectional and a longitudinal study. Both studies were conducted in the Dande Health and Demographic Surveillance System (Dande HDSS) area, implemented by the Health Research of Angola (CISA) in Bengo province, northern Angola. The main activity is agriculture and maize and cassava are the main crops grown in the region. Other activities include also fishing, charcoal exploitation and stone and sand extraction (39). STHs, schistosomiasis and malaria are endemic in the region, as reported in a community-based study conducted in 2010. (43). The main causes of death in under-five children between 2009 and 2012 in the verbal autopsy were intestinal infectious diseases, malnutrition and acute respiratory infections (186).

The cross-sectional study was conducted to identify the most frequent pathogenic agents of diarrhoea in children under-five attending the Bengo General Hospital (research question 1, paper I, published). Then, additional methodological techniques were performed to address the molecular characterization of rotavirus before the national vaccine introduction (research question 2, paper II, published); and the molecular characterization of *Giardia lamblia* (research question 3, paper III, published). Methodology of the cross-sectional study is described in Figure 18.

Finally, to investigate if giving albendazole or treating intestinal parasites after diagnosis at two different levels (individual and household) contributes to changing nutritional outcomes of children between 2 and 5 years (research question 4, paper IV, submitted), a longitudinal study was performed during two years, through a four-arm randomised controlled trial (trial registration ISRCTN-72928001).

1.7.1. Paper I

A cross-sectional hospital-based study was conducted between September 2012 and December 2013 in the Bengo General Hospital (BGH), the referral hospital for the Bengo province, in Caxito, Bengo province. Children under five years, with diarrhoea (114), attending the paediatric emergency unit or the hospital outpatient unit, were invited to participate in the study (Figure 18). Those with history of antibiotic and antiparasitic drug in the previous ten days were excluded to avoid false negatives. Informed consent was obtained from caregivers.

A survey to collect sociodemographic and clinical data was performed. Anthropometric measurements were assessed to calculate anthropometric indices (HAZ, WAZ, WHZ) using ANTHRO software, and according to WHO Child Growth Standards. Malnutrition was classified as mild, moderate and severe. Children with oedema were included in the severe level of acute malnutrition. One stool sample per child was requested to identify enteric viruses, bacteria and parasites, according to the laboratory methods described in the methodology section of paper I (please, see results section of this thesis, 2.1). Laboratory results were provided to the clinical staff of the hospital no more than a day after examination, Figure 19.

Data were analysed using IBM SPSS software. The χ^2 test was used for comparison of the categorical variable proportions. For tables with expected cell frequencies of less than 5, the Fisher exact test was applied. A P value of less than 0.05 was deemed statistically significant. The independent variables that were significantly associated with each response at a significance level of 0.1 were included in a multiple logistic regression model. The goodness of fit was based on Hosmer and Lemeshow test, considering a P value greater than 0.05. Adjusted associations were expressed in odds ratio (OR) and respective 95% confidence intervals (CIs).

The study was approved by the Ethics Committee of the Angolan Ministry of Health and the Ethics Committee of the Institute of the Hygiene and Tropical Medicine.

1.7.2. Paper II

In this study, only children who tested positive for rotavirus (immunochromatographic rapid test) in the previous study (paper I) investigating the most frequent agents of diarrhoea were included – Figure 18.

In Angola, stool samples were preserved in guanidine thiocyanate solution until RNA extraction. Then, stools were transported to the Institute of the Hygiene and Tropical Medicine for viral RNA extraction, reverse transcription, VP6 sub-grouping, G and P-genotyping, molecular characterization of rotavirus strains by phylogenetic analysis. Methodological aspects of these procedures are described in detail in the material and methods section of paper I (point 2.2).

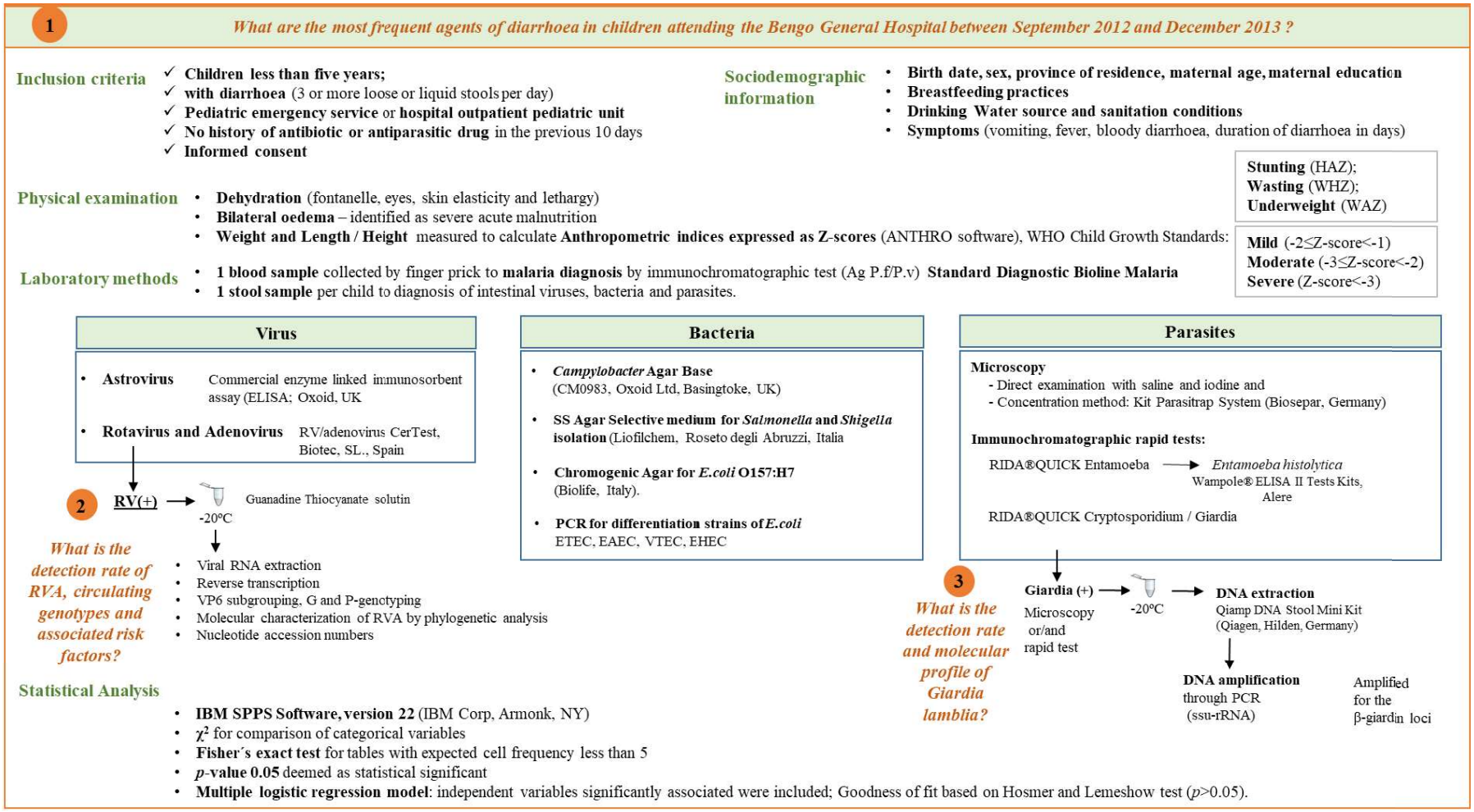

Figure 18. Methodological framework of the cross-sectional study

For statistical analysis, absolute (n) and relative frequency (%) were used for descriptive statistics of categorical and ordinal variables, and the mean and standard deviation (SD) were presented in the case of continuous variables. The chi-squared (χ^2) test or Fisher's exact test (for tables with expected cell frequencies less than 5) were used to compare categorical variable proportions (gender, group age, province of residence, settlement type, maternal literacy and education level, drinking water source and treatment method, sanitation facilities, breastfeeding, malnutrition and clinical symptoms) between RVA-positive and RVA-negative children. A p-value less than 0.05 was considered to be significant and associations were expressed in odds ratio (OR) and respective 95% confidence intervals (95% CI). Student t-test was applied to compare the mean age (in months) between RVA-positive and RVA-negative children. When the p-value was less than 0.05, the mean age between the two groups were considered significantly different.

1.7.3. Paper III

In this study, only children with stool samples positive for *Giardia lamblia* (through microscopy or using the antigen rapid test) in the previous study (aiming to identify the most frequent agents of diarrhoea, paper I) were included. Positive stool samples were preserved at -20ºC for DNA extraction, DNA amplification, and DNA sequence analysis – Figure 18. Laboratory methods are described in detail in the Material and Methods section of Paper III (point 2.3).

1.7.4. Paper IV

This is a four-arm randomised controlled trial conducted between December 2013 and January 2017 in the Health and Demographic Surveillance System (HDSS) (187) . This area includes Caxito, Mabubas, Úcua and a small part of Kicabo communes, located in Dande Municipality, Bengo, Angola (187).

In a first stage, between December 2013 and December 2014, children were recruited in three outpatient health units, namely: Hospital Geral do Bengo (outpatient unit), Hospital Municipal do Dande (outpatient unit) and Posto Médico o Bom Samaritano.

Children aged 20-36 months, living in the Dande Health and Demographic Surveillance System study area, with no history of antibiotic/antiparasitic drugs in the previous ten days, were invited to participate in the study. A sociodemographic survey was applied

and anthropometric assessment was performed. A single stool sample was requested for the microscopic detection of intestinal parasites. Then, after delivering the stool sample, those who were infected with at least one pathogenic intestinal parasite were invited to be included in the study for a two year of follow-up period.

As shown in Figure 19, 121 children were included and randomly allocated to one of the four arms (1:1:1:1): Arm 1 (A1) - to receive a single dose of ALB 400 mg once a year at individual level; Arm 2 (A2) - to receive a single dose of ALB 400 mg once a year at household level; Arm 3 (A3) - screening of pathogenic intestinal parasites every four months and treatment of positive results at individual level; in Arm 4 (A4) - screening of pathogenic intestinal parasites every four months and treatment of positive results at household level. Participants and clinical trial staff were aware of the group to which children were assigned.

Community follow-up (Fu) was performed at 4th (Fu1), 8th (Fu2), 12th (Fu3), 16th (Fu4), 20th (Fu5) and 24th months (Fu6) after inclusion of each child. The primary outcomes were growth assessed by height, HAZ, WHZ and WAZ at baseline, 4, 8 12, 16, 20 and 24 months of follow-up.

Sample size calculation was explored using GLIMMPSE software and 152 participants with intestinal parasitic infections was required (38 per arm). For more details, please see the appendix of Paper IV (point 2.4).

Intention-to-treat (ITT) analysis is widely recommended as the preferred analysis, as it avoid bias associated with non-random loss, in order to fully preserve the huge benefit of randomization (188). Thus, all randomised participants were included, after a missing values treatment (189). Analyses to primary outcomes started describing the proportion of subjects with missing by arms and choosing different methods to handle missing data. A special attention was given to height due to facility in terms of interpretation. In cases where the missing value was flanked by valid observations, interpolation was used to height, since there is a monotonic increasing in their values. For the remaining values, we performed multiple imputation using the Expectation Maximization, identifying the most plausible mechanism underlying our data. Missing Completely at Random (MCAR) was tested using Little´s test (188, 190).

Initially, nonparametric rank-based methods were explored in the nparLD (R software package) (191) to provide a response to the followed key questions for each primary outcome: i) do the arms/treatments have the same effect?; ii) Is the time profile flat or there is a trend over the follow-up period?; iii) Are the effects of the treatments similar over time? This first rank-based approach is robust to outliers and present a competitive performance for small sample sizes (191). However, other strategies are advantageous (189, 192). Thus, linear mixed effect models (LMM) and generalized estimating equations (GEE) for longitudinal data were explored to reinforce our findings, using packages (lme, nlme and geepack) (189, 192). Different correlation structures were also considered in several LMM and GEE models. Plots were explored using *gglopt2* package. Initial data analysis was done using IBM SPSS Software, version 24 (IBM Corp, Armonk, NY, USA), and a more advanced modelling was performed using R Program.

Data statistical analysis are described in detail in methods section of Paper IV (point 2.4).

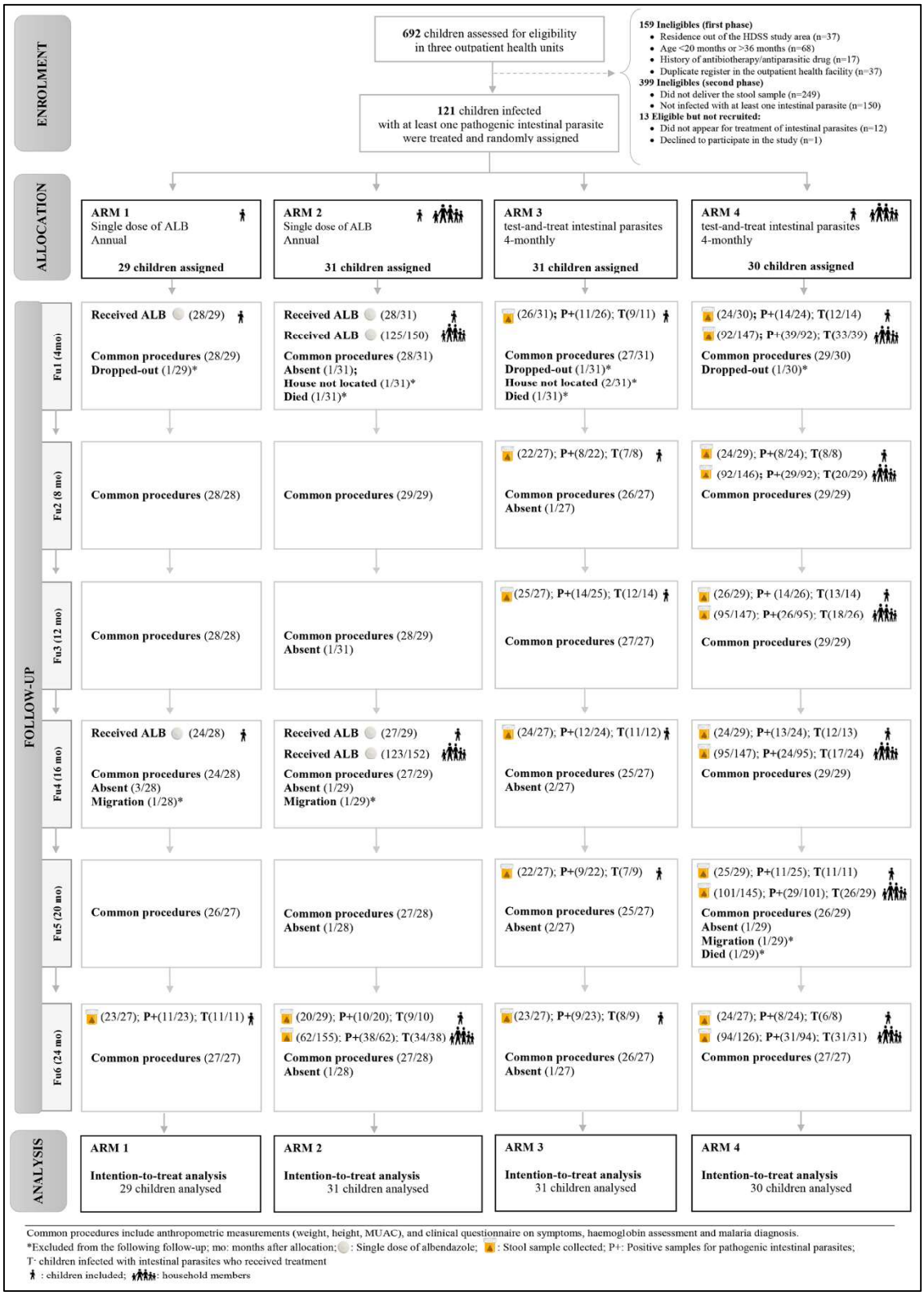

Figure 19. Consort flow-chart of the four-arm randomised controlled trial

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2. RESULTS

2.1 Paper I. Etiology of diarrhoea in children younger than 5 years attending the Bengo General Hospital in Angola

Reference:

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Etiology of Diarrhea in Children Younger Than 5 Years Attending the Bengo General Hospital in Angola

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Background: Diarrheal disease is among the leading causes of death in children younger than 5 years, especially in developing countries. The aim of this study was to investigate the most frequent etiological agents of diarrhea and its associated factors in children younger than 5 years attending the Bengo General Hospital in Angola.

Methods: From September 2012 through December 2013, stool samples were collected from 344 children presenting with diarrhea to investigate the presence of viral, bacterial and parasitic agents. Relevant sociodemographic and clinical data were obtained from parents and caregivers.

Results: An enteric pathogen was detected in 66.6% of stool samples: *Cryptosporidium* spp. (30.0%), rotavirus (25.1%), *Giardia lamblia* (21.6%), diarrheagenic *Escherichia coli* (6.3%), *Ascaris lumbricoides* (4.1%), adenovirus (3.8%), *Strongyloides stercoralis* (3.5%), astrovirus (2.6%), *Hymenolepis nana* (1.7%), *Entamoeba histolytica/dispar* (0.9%), *Taenia* spp. (0.6%), *Trichuris trichiura* (0.3%) and *Entamoeba histolytica* (0.3%). Children younger than 12 months were more frequently infected with *Cryptosporidium* spp. compared with older children (age: 12–59 months), independently of sex, season, lethargy and wasting [odds ratio (OR): 3.5, 95% confidence interval (95% CI): 2.0–6.2]. Age (OR: 5.0, 95% CI: 2.6–9.3), vomiting (OR: 2.7, 95% CI: 1.5–4.8) and type of admission (inpatients, OR: 0.5, 95% CI: 0.3–0.9) were significantly associated with rotavirus infection.

Conclusions: This study demonstrates high rates of infection with an enteric pathogen, particularly in children younger than 12 months, emphasizing the need to address diarrheal disease in this age group.

Key Words: diarrhea, etiology, Angola

(*Pediatr Infect Dis J* 2016;35:e28–e34)

Diarrhea is among the leading causes of death in children younger than 5 years, especially in developing countries where it is often associated with malnutrition, either as a cause or a consequence.[1,2] Diarrhea represents a symptom of gastrointestinal infections caused by bacteria, viruses, protozoa and helminths, and the route of transmission is usually fecal-oral or from person to person.[3] In some cases, infection results from skin penetration by infective larvae in contaminated soil. Poor hygiene conditions, lack of access to treated drinking water and inadequate sanitation foster high rates of transmission.[4] Several studies have shown that rotavirus (RV), norovirus, adenovirus and astrovirus; the protozoa *Giardia lamblia* and *Entamoeba histolytica*; the bacteria *Escherichia coli*, *Shigella* spp. and *Campylobacter* spp. are the most frequent diarrhea-associated agents.[3,5–8]

Angola has the highest under 5 mortality rate (167 deaths per thousand live births).[9] After malaria, diarrheal disease is considered the second most frequent disease in the country,[10] with one of the highest annual rates of child deaths being attributable to diarrhea (19.700 deaths).[3] Furthermore, the prevalence of chronic and acute malnutrition (29.0% and 8.0%, respectively) represents also a national concern.[11,12]

A community survey conducted in the Bengo province, Angola, identified high-prevalence estimates for geohelminths (22.6%), malaria (18.4%) and schistosomiasis (10.0%) in preschool children.[13] According to data from the Bengo General Hospital (BGH), the province's referral hospital, 14.9% of admissions of children younger than 5 years was because of diarrhea, the third cause of death in the hospital during 2009, after malaria and lower respiratory infections (unpublished data). Given the paucity of relevant data, this study aims to identify the most frequent etiological agents of diarrhea in children younger than five years attending the BGH.

MATERIALS AND METHODS

Study Design

A cross-sectional study was conducted between September 2012 and December 2013 in the BGH, located in Caxito, Dande Municipality, capital of Bengo province, 60 km northeast of Luanda. The climate is characterized by 2 seasons: a rainy (from October to April) and a dry season (from May to September).[14] The majority of patients attending the hospital live in the province, whereas some patients come from Luanda province given its proximity.

All the children younger than 5 years with diarrhea (3 or more loose or liquid stools per day),[3] attending the pediatric emergency service (inpatients and outpatients) or the hospital outpatient pediatric unit, were invited to participate in the study. Those receiving an antibiotic or antiparasitic drug within the preceding 10 days were excluded to avoid false-negative results.[3]

Parents or legal guardians of children meeting the inclusion criteria were interviewed by clinical staff. The survey included sociodemographic information (age in months, sex, province of residence, maternal age in years and maternal education); data on breastfeeding practices (exclusive breastfeeding was considered when the infant received only breast milk without addition of other liquids or solids, with the exception of oral rehydration solution, or drops/syrups of vitamins, minerals or medicines)[15]; water source and method of treatment; sanitation conditions (access to a private

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or shared latrine with or without water). Parents or legal guardians were asked about the duration of diarrhea (in days) and the presence of symptoms in the previous 10 days (vomiting, fever and bloody diarrhea). Physical examination of children was conducted to assess signs of dehydration (fontanelle, eyes, skin elasticity and lethargy). Weight and length/height were measured according to the standard procedures and used to calculate anthropometric indices expressed as Z score for each child with ANTHRO software: weight-for-age (underweight), weight-for-height (wasting) and height-for-age (stunting). Malnutrition was classified as mild ($-2 \leq Z$ score < -1), moderate ($-3 \leq Z$ score < -2) and severe (Z score < -3).[16] Children with symptoms of bilateral pitting edema were diagnosed as suffering from severe acute malnourishment.[17]

Sample Collection

A blood sample collected by finger prick was used for malaria diagnosis. A single stool sample per child was collected in a sterile container provided by clinical staff.

Laboratory Methods

Figure 1 shows the different laboratory diagnostic methods used for enteric pathogens detection (virus, parasites and bacteria) and the number of samples processed and those not processed because of insufficient stool amount. Once delivered at the laboratory, stool samples were immediately processed for pathogen detection with the adequate amount, in accordance with each manufacturer´s instructions.

RV and adenovirus were detected using rapid qualitative immunochromatographic assay (RV/adenovirus CerTest, Biotec S.L., Spain). Astrovirus was detected by a commercial enzyme-linked immunosorbent assay (ELISA; Oxoid, United Kingdom).

Microscopic detection of intestinal parasites was performed through direct examination with saline and iodine and a concentration method using the Kit Parasitrap-System (Biosepar, Germany). Immunochromatographic rapid tests were used to detect *Entamoeba* spp. (RIDAQUICK *Entamoeba,* R-Biopharm, Darmstadt, Germany), *G. lamblia* and *Cryptosporidium parvum*. (RIDAQUICK *Cryptosporidium/Giardia* Combi, R-Biopharm) antigens in stools. Positive stool samples for *Entamoeba* (by rapid test and/or microscopy) were then subjected to ELISA (Thermo Fisher Scientific) for the detection of *E. histolytica* (Wampole® ELISA II Test Kits, Alere™, Waltham, MA). The presence of malaria parasites was carried out by rapid immunochromatographic test (Standard Diagnostics Bioline Malaria Ag *P.f/P.v*, Standard Diagnostics Inc., Republic of Korea).[18]

Bacterial agents were studied by conventional solid selective culture for *Salmonella* spp. and *Shigella* spp. (Salmonella–Shigella Agar, Liofilchem, Roseto degli Abruzzi, Italy),[19] *Campylobacter jejuni* (Campylobacter Agar Base, product CM0983, Oxoid Ltd, Basingstoke, UK)[20] and *E. coli* (Chromogenic Agar for *E. coli* O157:H7, Biolife, Italy). After 12 hours of incubation, suspicious colonies for *Salmonella* spp., *Shigella* spp. and *E. coli* were confirmed using API20E (Biomerieux, Marcy-l'Etoile, France). An agglutination test on chromogenic agar was performed for *E. coli* O157:H7 serological confirmation of suspected colonies. Further differentiation of the diarrheagenic strains of *E. coli* was performed by polymerase chain reaction (PCR): enteropathogenic, enterotoxigenic (ETEC), enteroaggregative (EAEC), verotoxigenic and enterohemorrhagic.[21]

Ethical Considerations

The study protocol was approved by the Ethics Committee of the Angolan Ministry of Health and the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Portugal. Informed and voluntary consent was obtained from parents or legal guardians before the inclusion of each child. Parasitological (microscopy and rapid tests) and viral (rapid tests) test results were provided to the hospital clinical staff no more than a day after examination to ensure appropriate patient clinical management.

Statistical Analysis

Data were analyzed using IBM SPSS software, version 22 (IBM Corp, Armonk, NY). The χ^2 test was used for comparison of the categorical variable proportions. For tables with expected cell frequencies of less than 5, the Fisher exact test was applied. A P value of less than 0.05 was deemed statistically significant. The independent variables that were significantly associated with each response at a significance level of 0.1 were included in a multivariate logistic regression model. The goodness of fit was based on Hosmer and Lemeshow test, considering a P value greater than 0.05. Adjusted associations were expressed in odds ratio (OR) and respective 95% confidence intervals (CIs).

RESULTS

Three hundred forty-four children were included in the study: 53.0% male and 47.0% female (Table 1), with a mean age of 15.5 months ± 12.4 standard deviation (SD). The prevalence of exclusive breastfeeding in the first 6 months of life was 50.9% (28/55), and the mean age of weaning was 4.93 months ± 2.07 SD in children younger than 6 months and 15.05 months ± 6.57 SD in children aged between 6 and 24 months. The most frequent drinking water source was the river (27.0%), and bleach was the most commonly used method of water treatment. In terms of sanitation, 20.3% of households did not have a latrine (Table 1).

Among the children included, 52.3% were inpatients (Table 2). The mean duration of reported diarrheal episodes was 3.10 days ± 1.9 SD. Fever was the most frequent symptom reported (74.7%), followed by vomiting (51.5%) and bloody diarrhea (7.0%; Table 2). Sixty-one percent of children were underweight, 54% were stunted and 51% were wasted (Table 2). Nine children (9/289) were positive for *Plasmodium falciparum*.

An enteric pathogenic agent was identified in 66.6% of the total stool samples: in 41.9% of the samples, a single agent was detected, whereas in 24.7%, 2 or more pathogenic agents were identified. *Cryptosporidium* spp. (30.0%), RV (25.1%) and *G. lamblia* (21.6%) were the most frequent pathogenic agents. *Salmonella* spp., *Shigella* spp. and *Campylobacter* spp. were not isolated in any of the analyzed samples (Table 3).

In the univariate analysis, infection with RV was more frequent in children younger than 12 months than in older children aged 12–59 months ($P < 0.001$) and in inpatients compared with outpatients ($P = 0.002$; Table 4). Furthermore, RV infection was associated with vomiting ($P < 0.001$) and with wasting (Table 4). Conversely, RV was less frequent in children with stunting than in children without stunting ($P = 0.002$; Table 4). The multivariate analysis confirmed that RV infection was more frequent in younger children aged 0–12 months compared with older children aged 12–59 months (OR: 5.0, 95% CI: 2.6–9.3). Moreover, type of admission (OR: 0.5, 95% CI: 0.3–0.9) and vomiting (OR: 2.7, 95% CI: 1.5–4.8) continued to be associated with RV infection (Table 5).

Infection with *Cryptosporidium* spp. was more frequent in boys ($P = 0.047$), in children younger than 12 months compared with older children ($P < 0.001$) and more prevalent in the rainy season compared with dry season ($P = 0.037$; Table 4). Only age (OR: 3.5, 95% CI: 2.0–6.2) was significantly associated with *Cryptosporidium* spp. in the multivariate model fitted (Table 5).

Ten children (2.9%) were positive for *E. histolytica/dispar* by antigen rapid test (5 were also positive by microscopy). ELISA was performed on stool samples of 7 children of which 1 sample was confirmed as *E. histolytica* (0.3%; Fig. 1).

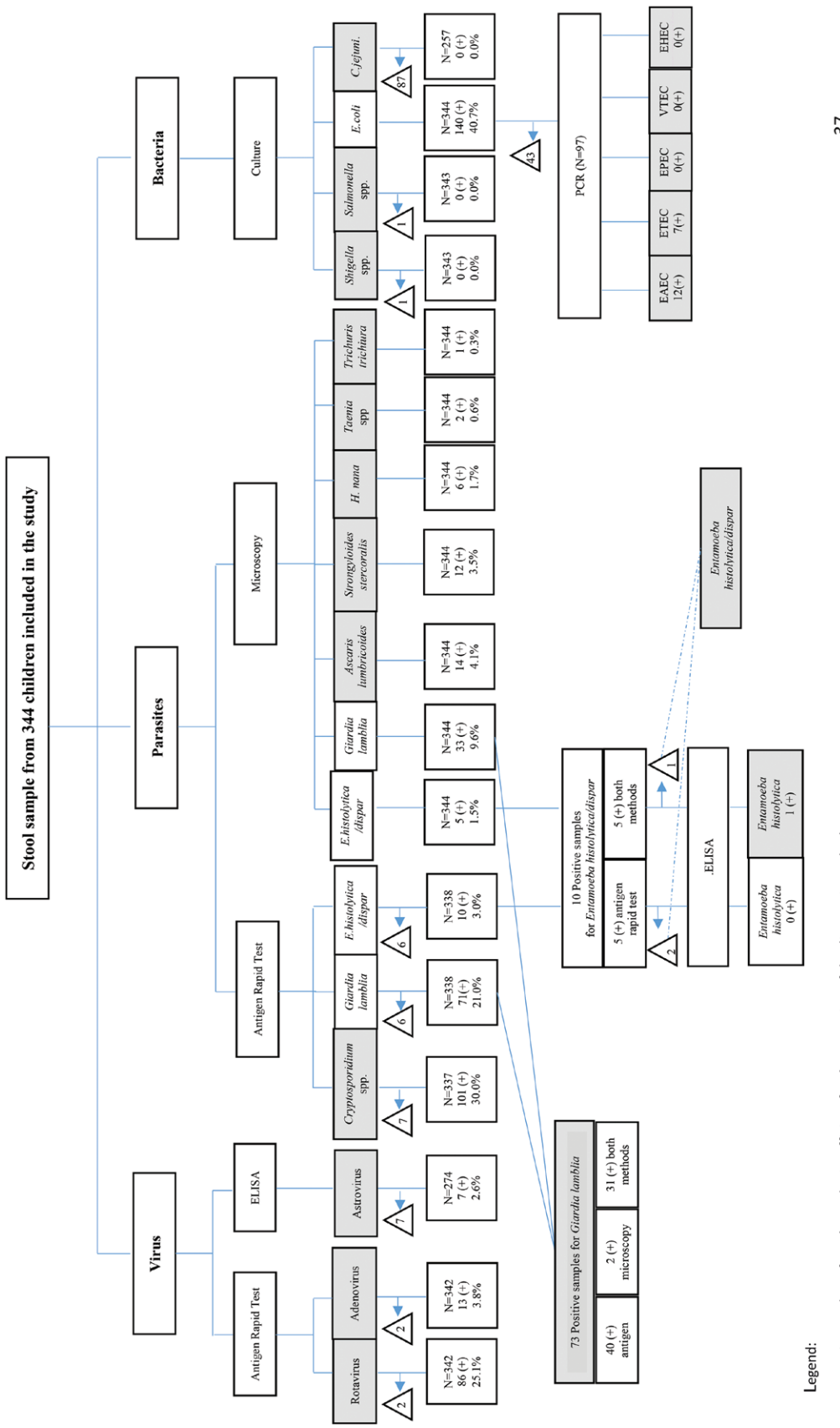

FIGURE 1. Laboratory diagnostic methods used for the detection of enteric pathogens (virus, parasites and bacteria) in the stool samples of the children with diarrhea included in the study.

TABLE 1. Sociodemographic Data of Children Younger Than 5 Years Included in the Study and Data on Breastfeeding, Maternal Age, Maternal Education, Drinking Water Source, Water Treatment Method and Sanitation

	N	n (%)
Age (mo)	344	
0–6		55 (16.0)
6–12		132 (38.4)
12–24		94 (27.3)
24–59		63 (18.3)
Sex	344	
Male		184 (53.0)
Female		160 (47.0)
Province	344	
Bengo		257 (83.4)
Luanda		57 (16.6)
Breastfeeding		
0–6 mo	55	
Exclusive		28 (50.9)
Complementary		25 (45.5)
Already weaned		1 (1.8)
Never		1 (1.8)
6–24 mo	226	
Exclusive		9 (4.0)
Complementary		172 (76.1)
Already weaned		44 (19.5)
Never		1 (0.4)
Maternal age	299	
13–20		55 (18.4)
20–30		163 (54.5)
30–49		81 (27.1)
Maternal education	339	
Without		65 (18.9)
Basic education		221 (64.2)
High school		48 (14.0)
Superior		5 (1.5)
Drinking water source	344	
River		93 (27.0)
Tap in the yard		84 (24.4)
Private tank		84 (24.4)
Public tap water		47 (13.7)
Piped water		21 (6.1)
Irrigation channel		5 (1.5)
Mineral water		4 (1.2)
Borehole		3 (0.9)
Tubewell		3 (0.9)
Water treatment method	344	
No		137 (39.8)
Yes		207 (60.2)
Bleach		170 (82.1)
Boiling		30 (14.5)
Alum stone		6 (2.9)
Filter		1 (0.5)
Sanitation	344	
Without latrine		70 (20.3)
With latrine		274 (79.7)
Private with water		100 (36.5)
Private without water		47 (17.2)
Shared with water		60 (21.9)
Shared without water		67 (24.5)

TABLE 2. Clinical Information of Children Younger Than 5 Years With Diarrhea Attending the Bengo General Hospital: Type of Admission, Reported Symptoms, Signs of Dehydration and Malnutrition (Underweight, Wasting and Stunting)

	N	n (%)
Type of admission	344	
Inpatient		180 (52.3)
Outpatient		164 (47.7)
Reported symptoms	344	
Fever		257 (74.7)
Vomiting		177 (51.5)
Diarrhea with blood		24 (7.0)
Signs of dehydration		
Reduced skin elasticity	320	14 (4.4)
Sunken fontanelle	322	49 (15.2)
Sunken eyes	331	77 (23.3)
Lethargy	327	95 (27.6)
Malnutrition		
Underweight	334	
Eutrophic		130 (38.9)
Mild		89 (26.6)
Moderate		65 (19.5)
Severe		50 (15.0)
Wasting	334	
Eutrophic		164 (49.1)
Mild		65 (19.5)
Moderate		37 (11.1)
Severe		68 (20.4)
Stunting	334	
Eutrophic		152 (45.5)
Mild		74 (22.2)
Moderate		53 (15.9)
Severe		55 (16.5)

ELISA was not performed in the remaining 3 stool samples because of their insufficient quantity and were therefore classified as *E. histolytica/dispar* (Table 3).

Hymenolepis nana was identified in 6 children, and all of them were stunted (Table 4). Maternal education and the presence of vomiting were significantly associated with *Strongyloides stercoralis* infection (Tables 4 and 5) independently of the considered confounders (OR: 0.2, 95% CI: 0.1–0.7; Table 5). No age differences were observed for *Ascaris lumbricoides*, *S. stercoralis*, *H. nana*, *Taenia* spp. and *Trichuris trichiura* infections. However, considering helminth infections collectively (N = 34), older children (age: 12–59 months) were more frequently infected than younger children (age: 0–12 months; $P = 0.019$).

The presence of *E. coli* was confirmed in 140 of 344 stool cultures. Among these, PCR was not performed on 43 samples because of an insufficient amount of stool (Fig. 1). The PCR confirmed 19 of 97 (19.6%) to be pathogenic: 12 of 97 (12.4%) EAEC and 7 of 97 (7.2%) ETEC (Table 3).

Among children younger than 6 months, there was no significant difference in the proportion of infection by any pathogenic agent between those who were and were not exclusively breastfed. Also, no significant difference was found between children using treated or untreated water and between the groups with or without a latrine.

DISCUSSION

At least 1 pathogenic agent was isolated in 66.6% of the study samples, supporting the importance of infectious causes of diarrheal disease in children younger than 5 years in this region of Angola. This percentage was higher compared with that obtained in a Mozambican hospital-based study (42.2%) but similar to a study conducted in Tanzania (67.1%).[6,22] Our study confirmed a high rate of infection caused especially by parasitic and viral agents, whereas bacterial agents were the less common.

The prevalence of RV (25.1%) was similar to estimates obtained in studies conducted in the Republic of Ivory Coast (28.6%),[23] but lower compared with a study from Huambo province, Angola (37.0%).[24] Some studies demonstrated an increase in childhood diarrhea hospitalizations worldwide because of RV: from 22% (between 1986 and 1999) to 39% (2000 and 2004).[25] Likewise, recent data from The African Rotavirus Surveillance Network

TABLE 3. Frequency of Pathogenic Agents Isolated From Stool Samples of Children Younger Than 5 Years With Diarrhea Attending the Bengo General Hospital

	N	n (%)
Virus		
Rotavirus	342	86 (25.1)
Adenovirus	342	13 (3.8)
Astrovirus	274	7 (2.6)
Parasites		
Cryptosporidium spp.	337	101 (30.0)
Giardia lamblia	338	73 (21.6)
Ascaris lumbricoides	344	14 (4.1)
Strongyloides stercoralis	344	12 (3.5)
Hymenolepis nana	344	6 (1.7)
Entamoeba histolytica /dispar	344	3 (0.9)
Entamoeba histolytica	341	1 (0.3)
Taenia spp.	344	2 (0.6)
Trichuris trichiura	344	1 (0.3)
Bacteria		
Diarrheagenic *Escherichia coli*	301	19 (6.3)
EAEC	301	12 (4.0)
ETEC	301	7 (2.3)
Salmonella spp.	343	0 (0.0)
Shigella spp.	343	0 (0.0)
Campylobacter jejuni	257	0 (0.0)

in children younger than 5 years hospitalized with acute diarrhea revealed a prevalence of 35% in a year round studies from Cameroon, Ethiopia and Zimbabwe and 41% in children recruited over a 2-year period in Ghana, Kenya, Uganda and Zambia.[26] In contrast to the aforementioned studies, we included inpatients and outpatients in our study, and RV infections were significantly lower in outpatients (22.6%) than in inpatients (29.6%) (OR: 0.5; 95% CI: 0.3–0.9). This could explain the lower RV prevalence in our study compared with studies recruiting patients requiring hospitalization.

Our results also demonstrate that RV infection was significantly more frequent in children younger than 12 months (OR: 4.7, 95% CI: 2.7–9.3) compared with older children. This is in line with the literature, reporting that 80% of primary RV infections in developing countries occur among infants younger than 12 months, as opposed to estimates from developed countries where the median age of primary infection ranges from 9 to 15 months.[26,27]

A higher number of RV cases were registered in children with wasting (30.4%) when compared with well-nourished children (20.7%). Acute malnutrition indicates recent weight loss and may typically be associated with insufficient food intake or a higher incidence of infectious diseases such as diarrhea.[1] In fact, some studies suggest that malnutrition may result from infection but also is itself a cause.[2] Also, the presence of vomiting was strongly associated with RV infection. This was previously reported in Africa and specifically in Angola suggesting that RV is an important cause of dehydration.[24,28,29] Among the viral agents, adenovirus and astrovirus were the least frequent viral agents (3.8% and 2.6%, respectively), which is in line with previous findings.[30,31]

Cryptosporidium spp. (30.0%) and *G. lamblia* (21.0%) were the most frequent protozoa agents detected in stool samples. However, the prevalence of *Cryptosporidium* spp. in our results contrasts with those from at least 2 recently hospital-based studies: one conducted in Kenya (4%) and another study conducted in Mozambique (0.6%).[6,32] A higher prevalence was reported in the Democratic Republic of Congo, in both community and health center (14.8% and 22.2%, respectively) using modified acid-fast stain method.[33] A study comparing modified acid-fast stain and antigen rapid test methods reported a slightly higher positivity among the second method (11.3% vs. 15.3%).[34] For diagnosing *Cryptosporidium* spp., conventional microscopy requires an expert technician and depends on the number of oocysts per gram of stool.[35] In this study, the use of an immunochromatographic test for detecting *Cryptosporidium* antigen could explain the higher prevalence of infection compared with those using conventional microscopy.

Children younger than 12 months were 3.7 times more likely to be infected with *Cryptosporidium* spp. ($P < 0.01$) compared with older children. This follows the findings of a study carried out in Kenya where children younger than 12 months were 2.4 times more likely to be infected by *Cryptosporidium* spp.[36] However, higher prevalence estimates have also been reported among children aged 12–24 months.[32,37] The introduction of complementary food above 6 months of age and the increased contact with contaminated water may contribute to a higher prevalence of infection.[38]

TABLE 4. Proportions of Enteric Pathogenic Agents According to Clinical Information: Type of Admission, Vomiting, Lethargy, Wasting and Stunting

	Admission, n (%)		Vomiting, n (%)		Lethargy, n (%)		Wasting, n (%)		Stunting, n (%)	
	Outpatient	Inpatient	Yes	No	Yes	No	Yes	No	Yes	No
Rotavirus	29 (17.7)	57 (32.0)*	62 (35.4)	24 (14.4)*	29 (31.2)	57 (24.6)	51 (30.4)	34 (20.7)	34 (18.9)	51 (33.6)*
Adenovirus	5 (3.0)	8 (4.5)	7 (4.0)	6 (3.6)	3 (3.2)	10 (4.3)	7 (4.2)	5 (3.0)	6 (3.3)	6 (3.9)
Astrovirus	1 (0.8)	6 (4.0)	2 (1.5)	5 (3.7)	0 (0.0)	6 (3.4)	4 (3.1)	3 (2.2)	3 (2.0)	4 (3.5)
Cryptosporidium spp.	47 (29.0)	54 (30.8)	46 (26.9)	55 (33.1)	34 (37.4)	61 (26.6)†	56 (34.1)	42 (25.8)†	47 (26.7)	51 (33.8)
Giardia lamblia	41 (25.3)	32 (18.2)	33 (19.2)	40 (24.1)	26 (28.3)	45 (19.7)†	29 (17.6)	42 (25.8)†	37 (20.9)	34 (22.5)
Entamoeba histolytica/ dispar	1 (0.6)	2 (1.1)	0 (0.0)	3 (1.8)	0 (0.0)	3 (1.3)	0 (0.0)	3 (1.8)	2 (1.1)	1 (0.7)
Entamoeba histolytica	1 (0.6)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.4)	1 (0.6)	0 (0.0)	1 (0.6)	0 (0.0)
Ascaris lumbricoides	5 (3.0)	9 (5.0)	8 (4.5)	6 (3.6)	5 (5.3)	9 (3.9)	9 (5.3)	5 (3.0)	7 (3.8)	7 (4.6)
Strongyloides stercoralis	3 (1.8)	9 (5.0)	10 (5.6)	2 (1.2)*	1 (1.1)	8 (3.4)	8 (4.7)	3 (1.8)	5 (2.7)	6 (3.9)
Hymenolepis nana	2 (1.2)	4 (2.2)	3 (1.7)	3 (1.8)	0 (0.0)	6 (2.6)	4 (2.4)	2 (1.2)	6 (3.3)	0 (0.0)*
Taenia spp.	1 (0.6)	1 (0.6)	1 (0.6)	1 (0.6)	1 (1.1)	0 (0.0)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.7)
Trichuris trichiura	0 (0.0)	1 (0.6)	1 (0.6)	0 (0.0)	1 (1.1)	0 (0.0)	1 (0.6)	0 (0.0)	1 (0.5)	0 (0.0)
Helminths	11 (6.7)	23 (12.8)	22 (12.4)	12 (7.2)	7 (7.4)	23 (9.9)	21 (12.4)	11 (6.7)	18 (9.9)	14 (9.2)
Diarrheagenic Escherichia coli	8 (5.8)	11 (6.8)	7 (4.4)	12 (8.5)	6 (7.1)	13 (6.5)	11 (7.6)	8 (5.5)	12 (7.5)	7 (5.4)
EAEC	6 (4.3)	6 (3.7)	4 (2.5)	8 (5.7)	5 (5.9)	7 (3.5)	8 (5.5)	4 (2.7)	9 (5.6)	3 (2.3)
ETEC	2 (1.4)	5 (3.1)	3 (1.9)	4 (2.8)	1 (1.2)	6 (3.0)	3 (2.1)	4 (2.7)	3 (1.9)	4 (3.1)

*P value < 0.05.
†0.05 < P value < 0.1.

TABLE 5. Sociodemographic and Clinical Characteristics Independently Associated With Infection by Rotavirus, *Cryptosporidium* spp. and *Strongyloides stercoralis* and Helminths Among Children With Diarrhea

Dependent Variable	Independent Variables	Unadjusted OR	95% CI	Adjusted OR	95% CI
Rotavirus*	Age†	4.747	2.645–8.519	4.969	2.650–9.319
	Vomiting‡	3.269	1.921–5.564	2.677	1.503–4.771
	Admission§	0.456	0.274–0.759	0.508	0.289–0.895
	Wasting‡	1.667	1.010–2.750	1.362	0.782–2.371
	Stunting‡	0.461	0.279–0.762	0.659	0.379–1.148
Cryptosporidium spp.¶	Sex‖	1.617	1.006–2.600	1.305	0.774–2.201
	Age†	3.868	2.288–6.539	3.531	2.022–6.166
	Season**	1.658	1.029–2.670	1.337	0.777–2.298
	Lethargy‡	1.643	0.981–2.752	1.620	0.909–2.887
	Wasting‡	1.494	0.927–2.407	1.489	0.886–2.500
Giardia lamblia††	Lethargy‡	1.611	0.921–2.816	1.770	0.991–3.161
	Wasting‡	0.072	0.361–1.047	0.734	0.421–1.279
Strongyloides stercoralis‡‡	Vomiting‡	4.940	1.066–22.891	3.198	0.656–15.590
	Maternal Education‡	0.153	0.043–0.540	0.188	0.052–0.678
Helminths§§	Age†	0.421	0.201–0.880	0.499	0.229–1.087
	Maternal Education‡	0.462	0.207–1.029	0.552	0.239–1.274
	Admission§	0.491	0.231–1.041	0.554	0.236–1.300

*Adjusted for age, vomiting, wasting and stunting.
†Reference class = 12–59 mo.
‡Reference class = no.
§Reference class = inpatient.
¶Adjusted for sex, age, season, lethargy and wasting.
‖Reference class = female.
**Reference class = dry season.
††Adjusted for lethargy and wasting.
‡‡Adjusted for vomiting and maternal education.
§§Adjusted for age, maternal education and admission.
OR indicates odds ratio.
The unadjusted OR and *P* values were calculated from univariate analysis and the adjusted OR from the fit of multivariate logistic regression.

Cryptosporidium spp. infection was more prevalent during rainy season, which is similar to the previous findings from Brazil and a meta-analysis that included 13 studies undertaken in Sub-Saharan Africa.[39,40] Rainfall in regions with low access to basic sanitation can increase the risk of water contamination with human or animal excreta.[39,40]

G. lamblia prevalence in our study was higher compared with studies conducted in Mozambique (2.5%), Cameroon (13.2%), Tanzania (14.0%) and Kenya (16%).[6,22,37,41] All the aforementioned studies applied microscopy for diagnosing *G. lamblia*. In our study, we identified *G. lamblia* by both methods (microscopy and the antigen rapid test) and found that more than half of the positive cases were detected only by the antigen rapid test. These differences could be explained by the fact that *G. lamblia* trophozoites are usually found in diarrheal stools, less resistant than cysts to environmental conditions and not detected if the microscopic analysis is not immediately conducted.[42] Moreover, the rapid diagnostic test are reported to be more sensitive than microscopy.[35]

Some studies identify *Cryptosporidium* spp. and *G. lamblia* as factors predisposing to significant growth impairment.[43] However, no significant differences were found between these protozoa agents and malnutrition. Longitudinal approaches are needed to investigate the relationship between infection and childhood growth.

Protozoa were more prevalent than helminths infections, probably because more than 80.0% of the sample was younger than 24 months. Furthermore, diarrhea is a common symptom of infection by protozoa.[35] Children whose mother had higher education levels presented lower odds of *S. stercoralis* infection (OR: 0.2, 95% CI: 0.1–0.7). This association between the mother's education and risk of intestinal parasites was previously reported in Mexico and is probably related to an adequate knowledge of hygiene practices.[44]

Despite the fact that several other African studies detected *C. jejuni* in prevalences ranging from 1.7% to 21.0%, we did not isolated this pathogen.[6,22,41,45] *Shigella* spp. and *Salmonella* spp. were also not isolated and are important bacterial agents usually linked to outbreaks.[6]

E. coli was the only potentially pathogenic bacterial agent isolated by our methodology. The importance of *E. coli* is well documented in diarrheal disease with EAEC and ETEC strains being more frequent than verotoxigenic or enterohemorrhagic among children in developing countries.[46] Suspicious colonies were isolated in 140 samples, but only 19 (6.3%) were confirmed as pathogenic: 12 EAEC (4.0%) and 7 ETEC (2.3%), with a higher prevalence in children younger than 6 months. A study conducted in Tanzania detected a higher prevalence of *E. coli* (22.9%) with EAEC predominating (14.6%) and also more prevalent in children younger than 6 months ($P < 0.05$).[47]

According to the literature, improvements in access to clean water and sanitation contribute to reduce diarrhea in African countries.[4] However, no significant association was observed between infectious agents and water sources, water treatment or latrine usage. When the interviews were performed, the water treatment procedures were not explored in detail to the parents, and for this reason, there may be insufficient data in these exposures to be associated with infection risk.

This study contain limitations: other diarrhea-associated pathogenic agents, such as norovirus and *Cyclospora* spp., fell beyond the scope of this research project. Collecting 3 instead of only 1 stool specimen would increase the microscopy detection rate of *G. lamblia*, as for other parasites, but parental collaboration would be much harder.[35] A further limitation of this study is that we included only children with diarrhea, but a case-control design comparing children with and without diarrhea would allow to explore potential associations between these exposures and the actual occurrence of the disease.

In conclusion, this study identified *Cryptosporidium* spp., RV and *G. lamblia* as the most frequent pathogens present in children younger than 5 years with diarrhea attending the BHG. Moreover, children younger than 12 months were more frequently infected with *Cryptosporidium* spp. and RV than older children. This reinforces the

importance of interventions for diarrhea control in the earlier months of life. In 2014, Angola included RV vaccine in the national immunization plan.[48] Surveillance of the RV disease in the next few years is going to be crucial for a better understanding of diarrhea-associated morbidity and mortality, but other diarrhea-associated agents need to be addressed. This study presents relevant information for health policy makers and emphasizes the importance of diagnosing the agents of diarrhea in young children as a part of them can be easily treated.

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Paper II. Characterization of rotavirus infection in children with acute gastroenteritis in Bengo province, Northwestern Angola, prior to vaccine introduction

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RESEARCH ARTICLE

Characterization of rotavirus infection in children with acute gastroenteritis in Bengo province, Northwestern Angola, prior to vaccine introduction

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Abstract

Background

Rotavirus group A (RVA) is considered the leading cause of pediatric diarrhea, responsible for the high burden of diarrheal diseases in sub-Saharan Africa. Despite recent studies, the existent data are scarce for some African countries like Angola, a country with one of the highest RVA-related death estimates. The aim of this study was to determine the RVA detection rate and circulating genotypes in children less than five years of age with acute gastroenteritis attended at the Bengo General Hospital in Caxito, Bengo province, Angola, before vaccine introduction.

Methods

Between September 2012 and December 2013, 342 fecal specimens were collected from children enrolled. Positive samples for RVA by immunochromatographic rapid test were G and P-typed by hemi-nested type-specific multiplex PCR, and subgrouped for the VP6 gene. VP4 and VP7 genes from a subset of samples were sequenced for phylogenetic analysis.

Results

During the study period, a high RVA detection rate was registered (25.1%, 86/342).

The age group most affected by RVA infection includes children under 6 months of age ($p<0.01$). Vomiting was highly associated with RVA infection (72.1%; $p<0.001$).

From the 86 RVA-positive samples, 72 (83.7%) were genotyped. The most prevalent genotype was G1P[8] (34/72; 47.2%), followed by the uncommon G1P[6] (21/72; 29.2%), and G2P[4] (9/72; 12.5%). Only two G-types were found: G1 (60/72; 83.3%) and G2 (11/72;

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15.3%). Among the P-genotypes, P[8] was the most prevalent (34/72; 47.2%), followed by P [6] (22/72; 30.6%) and P[4] (9/72; 12.5%). In the phylogenetic trees, the identified G and P-types clustered tightly together and with reference sequences in specific monophyletic groups, with highly significant bootstrap values (≥92%).

Conclusion

This pre-vaccination study revealed, for the first time for Bengo province (Angola), the RVA genotype profile, including phylogenetic relationships, and a high RVA detection rate, supporting the immediate introduction of a RVA vaccine in the national immunization programme.

Introduction

Rotavirus group A (RVA) remains the most important etiological agent of severe diarrhea in children under five years of age [1, 2], especially in remote areas with difficult or inexistent access to the healthcare infrastructure and inadequate domestic sanitation conditions [2, 3].

According to the global estimates from 2008, RVA was responsible for 453,000 deaths among children under five years of age each year, with African children accounting for more than 50% of the total [4], but a recent study showed a decline in this number, up to 215,000 deaths in 2013, the majority of them in India [5]. In the last decade, an increased research effort on epidemiology and disease burden of RVA infection has been made. Data obtained helped in the definition of more effective health policies, including the implementation of RVA vaccination. Two live attenuated RVA vaccines, the monovalent Rotarix (GlaxoSmithKline Biologicals, Belgium) and the pentavalent human-bovine reassortant RotaTeq (Merck, USA), were recommended by the World Health Organization (WHO) to be included in the national immunization programmes worldwide in 2009 [6]. Recently, a third RVA vaccine, the low-cost, live attenuated Rotavac (Bharat Biotech International, India) was licensed for use in India but not yet pre-qualified for the Global Alliance for Vaccines and Immunization (GAVI) market [7]. Both recommended vaccines require multiple dose administration (two doses for Rotarix and three for RotaTeq), the first to be administered between 6 and 15 weeks of age [8], and raise homo- and heterotypic immune response against RVA different strains [9]. The two vaccines have been proven to be effective worldwide, but lower efficacy was observed in low-income countries from Africa and Southern Asia [10, 11]. Among the several hypothesis to explain the differences in the immune response and consequent efficacy of these vaccines in low- *versus* high-income countries, RVA strains diversity, host genetic factors, malnutrition, host co-infection, deficient micronutrient ingestion, and interfering gut flora have been put forward [12–14].

Worldwide, there is a great genetic diversity of circulating RVA wild-type strains. Several studies conducted in Africa identified G1P[8] as the most frequent RVA strain, while the most common strains for high- and middle-income countries in the *pre* vaccination era, G2P[4], G3P[8], G4P[8], and G9P[8] [15], have also been identified, but to a much lesser extent [16, 17]. Noteworthy, is the emergence of uncommon strains, such as G1P[6], G8P[6], G6P[6], G8P[8], G12P[6], and mixed G and P-strains, in sub-Saharan Africa [18–21]. This picture is still far from completed for the majority of African countries, e.g. Angola. Despite the recent publication of an RVA epidemiology study in four provinces of Angola [22], present data on RVA prevalence and genotype distribution remain scarce.

Angola is a sub-Saharan African country with an estimated population of 25.8 million in 2014. The Angolan population is very young, with an average age of 20.6 years and a proportion of 47.3% aged 0–14 years [23]. Angola has one of the highest RVA attributable death rate in children under five years (5% of global total) [5]. Bengo province, situated in Northwestern Angola and close to the capital city, Luanda, has a population of about 357.000 individuals, with 56.3% of them living in rural settings [24]. The capital, Caxito, harbor the Bengo General Hospital (BGH), a reference hospital for the province. To reduce severe diarrheal disease, and preventing RVA-associated hospitalizations and mortality, Angola requested assistance from the GAVI in 2014 to introduce RVA vaccine in the national immunization program.

This study is part of a larger investigation regarding diarrhea burden in children under five years in the Bengo province, Angola [25], and aims to provide baseline information on RVA infection before vaccine introduction. This hospital-based study was established to investigate RVA prevalence and genotype distribution during a period of more than one year in children up to five years attended with acute gastroenteritis (AGE).

Material and methods

Setting or study site

This study was conducted at the Bengo General Hospital (BGH), located in Caxito, capital of Bengo province, 60 km northeast of the capital Luanda. Besides receiving patients from Bengo province, the BGH also attends patients from neighboring Luanda province. The climate of this province is subtropical, characterized by a rainy and warm season, from mid-September to mid-May, and a dry and cold season, from mid-May to mid-September.

Study design

This study was conducted at the BGH between September 2012 and December 2013 and is part of a cross-sectional study set up to investigate the most frequent etiological agents of diarrhea (viruses, parasites and bacteria) in children under five years of age [25]. Surveillance data was collected before rotavirus group A (RVA) vaccine introduction in the country. A total of 342 children with acute gastroenteritis AGE (diarrhea, with or without vomiting) attending the pediatric emergency room or the outpatient pediatric unit were enrolled and tested for RVA infection. During the sampling period, all children with AGE presenting at BGH, originating from Bengo province (n = 286) or Luanda province (n = 56), were included in this study. Diarrhea was defined as three or more loose or liquid stools per day [26]. Children receiving antibiotic or antiparasitic treatment within 10 days before the examination were excluded from the study. After informed consent was given by parents or legal guardians, sociodemographic variables [i.e. date of birth (n = 342), gender (n = 342), province of residence (n = 342), residence type (n = 250), maternal literacy (n = 337), education level of the mother (n = 272)], and information on breastfeeding practices (n = 240), drinking water source (n = 342), water treatment methods (n = 342) and sanitation conditions (n = 342) were obtained, whenever possible. Nutritional status was also assessed from anthropometric measurements (n = 332). Weight and length/height were measured according to standard procedures established by the World Health Organization (WHO) and used to calculate anthropometric indices expressed as individual *z* scores using ANTHRO software (version 3.2.2) [27]: weight-for-age (WAZ, underweight), weight-for-height (WHZ, wasting) and height-for-age (HAZ, stunting). Malnutrition was classified as mild ($-2 \leq z$ score < -1), moderate ($-3 \leq z$ score < -2) or severe (z score < -3) [28]. Children with symptoms of bilateral pitting edema were diagnosed as suffering from severe acute malnourishment as described in WHO procedures [29].

After physical examination, the clinical variables regarding symptoms associated to AGE were registered [e.g. duration of diarrhea in days (n = 333), vomiting (n = 342), fever

(n = 342), lethargy (n = 325), dehydration signs, such as depressed fontanelle (n = 320), sunken eyes (n = 329), skin elasticity (n = 318)]. Depending upon the severity of symptoms, each child was referred for regular follow-up treatment (oral rehydration, drugs, etc.) or hospitalization. The type of admission was registered [e.g. outpatient department (n = 164) or emergency unit (n = 178)].

Ethical considerations

The study protocol (including detailed working plan, epidemiological survey and informed consent forms) was approved by the National Ethics Committee of the Angolan Ministry of Health in Luanda and the Ethics Committee of the Institute of Hygiene and Tropical Medicine, in Lisbon, Portugal (Process number:12-2012-PN). As stated before, informed and voluntary written consent was obtained from parents or legal guardians of each child prior to inclusion in the study.

Sample collection and RVA antigen detection

Fecal specimens were collected in sterile containers provided by clinical staff. A total of 342 samples were screened locally for the presence of RVA and adenovirus serotype 40/41 antigens using a rapid qualitative immunochromatographic assay (Rotavirus + Adenovirus, CerTest Biotec S.L., Zaragoza, Spain), following the manufacturer's instructions. RVA-positive stool samples were preserved in guanidine thiocyanate solution until RNA extraction as described before [30].

Viral RNA extraction

Preserved stool samples were transported to the Institute of Hygiene and Tropical Medicine in Lisbon, Portugal for RVA genotyping. Viral RNA was extracted from 10% (w/v) stool suspensions using the innuPREP Virus RNA Kit (Analytik Jena AG, Jena, Germany), according to the manufacturer's instructions. The RNA was eluted in RNase-free water (60 μl) and stored at -80˚C until further use.

Reverse transcription, VP6 subgrouping, G and P-genotyping

Reverse transcription (RT) with random hexamers, to produce cDNA to be used as template in specific PCRs for the different genes, was carried out using a commercial kit (NZY First-strand cDNA Synthesis Kit, NZYTech, Lisbon, Portugal), according to the manufacturer's instructions. Briefly, 16 μl of the RNA eluate was denatured at 94˚C for 5 minutes, and quickly chilled on ice for 2 minutes, followed by the addition of 20 μl of NZYRT 2x Master Mix and 4 μl of NZYRT Enzyme Mix, to a final volume of 40 μl. The RT reaction was carried out at 25˚C for 10 minutes, and 50˚C for 30 minutes, being the inactivation made at 85˚C for 5 minutes. After the final addition of 2 μl of NZY RNase H (*E. coli*), the product was incubated at 37˚C for 20 minutes.

VP6 subgrouping was performed using conventional PCR, previously described in 2010 by Thongprachum et al. [31].

RVA G and P-genotyping were done using hemi-nested type specific multiplex PCRs, optimized to detect eight G-types (G1, G2, G3, G4, G8, G9, G10 and G11) and six P-types (P[4], P[6], P[8], P[9], P[10] and P[11]), as described previously [32–34]. The G- and P-genotypes were assigned according to the amplicon size visualized under ultraviolet light after electrophoresis on 2% agarose gels stained with ethidium bromide.

Molecular characterization of RVA strains by phylogenetic analysis

First-round PCR amplicons for VP7 and VP4 genes from a set of randomly selected G- and P-typed viruses from each detected genotype, as well as from non-typable strains, were sent for DNA sequencing (Sanger method) for further molecular characterization. DNA sequencing was performed using the corresponding first-round PCR primers by STAB VIDA (Caparica, Portugal).

After sequence editing using the BioEdit Sequence Alignment Editor version 7.1.3.0 [35], multiple sequence alignments were made with Clustal Omega (available at http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was performed with the MEGA 5.1 software [36], using a distance-based neighbour-joining method, based on the Kimura 2-parameter model [37]. Bootstrap values were calculated from 1,000 replicates [36].

Nucleotide sequence accession numbers

The GenBank/DDBJ/EMBL accession numbers for sequences obtained in this study are KP216531-KP216547, for VP4, and KP216548-KP216561, for VP7.

Statistical analysis

Data were analyzed using IBM SPSS software, version 22 (IBM Corp, Armonk, NY, USA). Absolute (n) and relative frequency (%) were used for descriptive statistics of categorical and ordinal variables, and the mean and standard deviation (SD) were presented in the case of continuous variables.

The chi-squared ($\chi 2$) test or Fisher's exact test (for tables with expected cell frequencies less than 5) were used to compare categorical variable proportions (gender, group age, province of residence, settlement type, maternal literacy and education level, drinking water source and treatment method, sanitation facilities, breastfeeding, malnutrition and clinical symptoms) between RVA-positive and RVA-negative children. A p-value less than 0.05 was considered to be significant and associations were expressed in odds ratio (OR) and respective 95% confidence intervals (95% CI). A multiple logistic regression model was applied for stunting (dependent variable), and included the independent variables age, gender, infection with RVA and infection by an enteric pathogen other than RVA (data from the cross-sectional study published before [25]). The goodness of fit was based on Hosmer and Lemeshow test, considering a *p* value greater than 0.05. Student´s *t*-test was applied to compare the mean age (in months) between RVA-positive and RVA-negative children. When the p-value was less than 0.05, the mean age between the two groups were considered significantly different.

Results

RVA detection rate and basic socio-demographic characterization of the studied population

From September 2012 to December 2013, 342 children under five years of age with acute gastroenteritis (AGE), attended at the Bengo General Hospital (BGH), were tested for rotavirus group A (RVA) and enteric adenovirus (AdV) type 40/41 infections. RVA detection rate was 25.1% (86/342), while for AdV type 40/41 was much lower, reaching 3.8% (13/342). Two children were shown to have mixed infection (0.6%, 2/342).

No association was found between infection by RVA and children gender (Table 1). The mean age was significantly lower for children with RVA infection as detected by the RVA antigen immunochromatographic assay (9.2±5.00 *versus* 17.6±13.40 months, $p<0.001$)—Table 1. The age distribution is significantly different between RVA infected and non-infected children

Table 1. Characteristics of the study sample.

		RV (+)	RV (-)	*p-value*	Total
		n (%)	n (%)		n (%)
Study sample		86 (25.1)	256 (74.9)		342 (100)
Gender	Male	49 (57.0)	134 (52.3)	0.456	183 (53.5)
	Female	37 (43.0)	122 (47.7)		159 (46.5)
Mean age (months)		9.2±5.00	17.6±13.4	**<0.001**	15.5±12.39
Group age (months)	[0–6[	21 (24.4)	34 (13.3)	**<0.001**	55 (16.1)
	[6–12[	48 (55.8)	84 (32.8)		132 (38.6)
	[12–24[	16 (18.6)	76 (29.7)		92 (26.9)
	[24–59]	1 (1.2)	62 (24.2)		63 (18.4)

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($p<0.001$, Table 1), with a positive association between infection and younger ages (<12 months).

Although BGH is a reference hospital for the province, 16.4% (56/342) of the attended children were residents of the neighbor Luanda province (Table 2). The vast majority of the enrolled children were living in an urban area (92.4%, 231/250), with only 7.6% (19/250) living in rural settlements (Table 2). When asked about maternal education, the majority of the respondents (65.0%, 219/337) declared as graduating basic education, only 14.2% (48/337) frequented the high school, and 1.5% (5/337) had a university degree. 19.3% (65/337) of the mothers never attended school. No significant association was found between level of maternal literacy or education and RVA infection or the age of children enrolled. Apart from age and age distribution, there was no statistically significant association between RVA infection and any other socio-demographic variable analyzed.

Drinking water source and treatment method

The majority of the households with children participating in the study (69.6%, 238/342) used water supplied by the Central Water Treatment Plant (CWTP) (Table 2). However, in 58.8% (140/238) of these households an additional treatment, including the use of bleach and alum stone, boiling and/or filtration, was applied before drinking. The consumption of water only treated at home or without any type of treatment was reported, respectively, for 19.0% (65/342) and 11.4% (39/342) of the households.

Among all the RVA positive cases, 43.0% (37/86) of children were drinking water from the CWTP with a further household treatment, 31.4% (27/86) were using water from CWTP without any additional treatment. In 16.3% (14/86) of the cases, it was applied only a household treatment and a minority of the children (9.3%, 8/86) drank untreated water. This distribution was not significantly different for RVA negative children. Comparing RVA-positive and -negative children of different age groups, no significant association was found concerning the drinking water source and the treatment method applied (Table 2).

Sanitation facilities

Regarding sanitation facilities, 79.5% (272/342) of the children had access to latrines, either private (53.7%, 146/272) or public (46.3%, 126/272). In a significant proportion of these latrines (41.9%, 114/272), there was no running water. So, for a high proportion of children, i.e. 20.5% (70/342), without access to any type of latrines, the most probable option could mean open defecation close to the households. The use of a public latrine with running water determined the highest RVA infection rate in children (35.6%, 21/59). On the other hand, the

Table 2. Sociodemographic profile of children with diarrhea attended at the Bengo General Hospital.

Variable	[0–12 [months		[12–24 [months		[24–59] months		[0–59] months	
	RV(+)	RV(-)	RV(+)	RV(-)	RV(+)	RV(-)	RV(+)	RV(-)
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Child gender (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
Male (N = 183)	39 (56.5)	72 (61.0)	9 (56.3)	34 (44.7)	1 (100.0)	28 (45.2)	49 (57.0)	134 (52.3)
Female (N = 159)	30 (43.5)	46 (39.0)	7 (43.8)	42 (55.3)	0 (0.0)	34 (54.8)	37 (43.0)	122 (47.7)
Province of residence (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
Bengo (N = 286)	54 (78.3)	100 (84.7)	14 (87.5)	64 (84.2)	1 (100)	53 (85.5)	69 (80.2)	217 (84.8)
Luanda (N = 56)	15 (21.7)	18 (15.3)	2 (12.5)	12 (15.8)	0 (0.0)	9 (14.5)	17 (19.8)	39 (15.2)
Settlement type[a] (N = 250)	N = 49	N = 90	N = 8	N = 57	N = 1	N = 45	N = 58	N = 192
Urban (N = 231)	44 (89.8)	81 (90.0)	8 (100.0)	54 (94.7)	1 (100.0)	43 (95.6)	53 (91.4)	178 (92.7)
Rural (N = 19)	5 (10.2)	9 (10.0)	0 (0.0)	3 (5.3)	0 (0.0)	2 (4.4)	5 (8.6)	14 (7.3)
Maternal literacy (N = 337)	N = 69	N = 115	N = 16	N = 74	N = 1	N = 62	N = 86	N = 251
No (N = 65)	14 (20.3)	20 (17.4)	2 (12.5)	13 (17.6)	0 (0.0)	16 (25.8)	16 (18.6)	49 (19.5)
Yes (N = 272)	55 (79.7)	95 (82.6)	14 (87.5)	61 (82.4)	1 (100.0)	46 (74.2)	70 (81.4)	202 (80.5)
Education level of the mother (N = 272)	N = 55	N = 95	N = 14	N = 61	N = 1	N = 46	N = 70	N = 202
Basic (N = 219)	46 (83.6)	78 (82.1)	11 (78.6)	48 (78.7)	1 (100.0)	35 (76.1)	58 (82.9)	161 (79.7)
High school (N = 48)	6 (10.9)	16 (16.8)	3 (21.4)	13 (21.3)	0 (0.0)	10 (21.7)	9 (12.9)	39 (19.3)
University (N = 5)	3 (5.5)	1 (1.1)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.2)	3 (4.3)	2 (1.0)
Water source from the CWTP[b] (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
Yes (N = 238)	51 (73.9)	80 (67.8)	12 (75.0)	50 (65.8)	1 (100.0)	44 (71.0)	64 (74.4)	174 (68.0)
No (N = 104)	18 (26.1)	38 (32.2)	4 (25.0)	26 (34.2)	0 (0.0)	18 (29.0)	22 (25.6)	82 (32.0)
Drinking water source/treatment[c] (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
From CWTP and not treated at home (N = 98)	20 (29.0)	32 (27.1)	6 (37.5)	21 (27.6)	1 (100.0)	18 (29.0)	27 (31.4)	71 (27.7)
From CWTP and treated at home (N = 140)	31 (44.9)	48 (40.7)	6 (37.5)	29 (38.2)	0 (0.0)	26 (41.9)	37 (43.0)	103 (40.2)
Only treated at home (N = 65)	11 (15.9)	25 (21.2)	3 (18.8)	16 (21.1)	0 (0.0)	10 (16.1)	14 (16.3)	51 (19.9)
Not treated (N = 39)	7 (10.1)	13 (11.0)	1 (6.3)	10 (13.2)	0 (0.0)	8 (12.9)	8 (9.3)	31 (12.1)
Sanitation facilities (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
Without latrine (N = 70)	12 (17.4)	24 (20.3)	2 (12.5)	14 (18.4)	0 (0.0)	18 (29.0)	14 (16.3)	56 (21.9)
Private latrine with running water (N = 99)	18 (26.1)	39 (33.1)	4 (25.0)	20 (26.3)	1 (100.0)	17 (27.4)	23 (26.7)	76 (29.7)
Private latrine without running water (N = 47)	11 (15.9)	12 (10.2)	3 (18.8)	14 (18.4)	0 (0.0)	7 (11.3)	14 (16.3)	33 (12.9)
Public latrine with running water (N = 59)	15 (21.7)	18 (15.3)	6 (37.5)	11 (14.5)	0 (0.0)	9 (14.5)	21 (24.4)	38 (14.8)
Public latrine without running water (N = 67)	13 (18.8)	25 (21.2)	1 (6.3)	17 (22.4)	0 (0.0)	11 (17.7)	14 (16.3)	53 (20.7)
Breastfeeding (N = 342)	N = 69	N = 118	N = 16	N = 76	N = 1	N = 62	N = 86	N = 256
Exclusive (N = 38)	14 (20.3)	20 (16.9)	0 (0.0)	3 (3.9)	0 (0.0)	1 (1.6)	14 (16.3)	24 (9.4)
Complementary (N = 202	54 (78.3)	85 (72.0)	14 (87.5)	45 (59.2)	0 (0.0)	4 (6.5)	68 (79.1)	134 (52.3)
Weaned (N = 98)	1 (1.4)	11 (9.3)	2 (12.5)	28 (36.8)	1 (100.0)	55 (88.7)	4 (4.7)	94 (36.7)
Never (N = 4)	0 (0.0)	2 (1.7)	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.2)	0 (0.0)	4 (1.6)
Malnutrition (N = 332)								
- Underweight *(weight for age z score)*								
WAZ $\leq$ -2SD (N = 113)	23 (33.8)	36 (31.3)	9 (56.3)	25 (35.2)	1 (100.0)	19 (31.1)	33 (38.8)	80 (32.4)
WAS > -2SD (N = 219)	45 (66.2)	79 (68.7)	7 (43.8)	46 (64.8)	0 (0.0)	42 (68.9)	52 (61.2)	167 (67.6)
- Wasting *(weight for height z score)*								
WHZ $\leq$ -2SD (N = 103)	26 (37.7)	32 (28.3)	5 (33.3)	26 (35.1)	1 (100.0)	13 (21.7)	32 (37.6)	71 (28.7)
WHZ > -2SD (N = 229)	43 (62.3)	81 (71.7)	10 (66.7)	48 (64.9)	0 (0.0)	47 (78.3)	53 (62.4)	176 (71.3)
- Stunting *(height for age z score)*								
HAZ $\leq$ -2SD (N = 107)	10 (14.5)	37 (32.7)*	4 (26.7)	33 (44.6)	0 (0.0)	23 (38.3)	14 (16.5)	93 (37.7)**

(*Continued*)

Table 2. (*Continued*)

Variable	[0–12 [months		[12–24 [months		[24–59] months		[0–59] months	
	RV(+)	RV(-)	RV(+)	RV(-)	RV(+)	RV(-)	RV(+)	RV(-)
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
HAZ > -2SD (N = 225)	59 (85.5)	76 (67.3)	11 (73.3)	41 (55.4)	1 (100.0)	37 (61.7)	71 (83.5)	154 (62.3)

[a]From the 342 households, only those from Centro de Investigação em Saúde de Angola (CISA) study area were included for analysis (N = 250).

[b] Water from the Central Water Treatment Plant (CWTP) included drinking water from taps in the yard, private tanks, public taps and piped water. Other water sources, as the river, irrigation channel and borehole/tubewell were considered not being from the CWTP.

[c] Water treatment methods at home included the use of bleach or alum stone, boiling and filtration.

* *p* value (x^2) = 0.006; OR = 0.348; 95%CI] 0.160–0.757[

** *p* value (x^2) <0.001; OR = 0.327; 95%CI] 0.174;0.612[

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lowest rate of infection is associated with the absence of latrine (20.0%, 14/70). Nonetheless, no significant difference was observed between the type of sanitation facilities (Table 2) and the rate of RVA infection, on the overall and when considering each age group per se.

Breastfeeding and nutritional assessment

Breastfeeding practice was reported for 240 (70.2%) children included in the study (of whom 38 were exclusively breastfed and 202 had complementary breastfeeding), while 98 children have already been weaned (28.7%) and 4 have never been breastfed (1.2%). RV infection was significantly more frequent among children who were being breastfed at the time of enrollment (82/240, 34.2%), compared to those that had already been weaned or had never been breastfed (4/102, 3.9%)–p-value <0.001; OR = 12.7; 95% CI [4.518;35.786]. In relation to nutritional assessment, 34.0% (113/332) of the enrolled children were moderate to severe underweighted, 31.0% (103/332) presented moderate to severe wasting and 32.2% (107/332) were moderate to severe stunted. Overall, the percentage of underweighted children (*z* score≤-2) was higher in children with RVA infection as compared to the non-infected (38.8% versus 32.4%, respectively, p = 0.280; CI [0.795; 2.209]), with a higher percentage of underweighted children among RVA infected children between 12–24 months compared to negative cases in the same age group (56.3% and 35.2%, respectively, p = 0.119; CI [0.786;7.116]) (Table 2). However, none of these differences proved to be significant. Similarly, the percentage of wasted children (*z* score≤-2) was higher in children with RVA infection compared to those with a negative result for RVA antigen detection (37.6% and 28.7%, respectively, p = 0.126; CI [0.89;2.513]), more evident for children under twelve months (p = 0.188), although also without significant difference. In contrast, the overall proportion of children stunted (*z* score≤-2) was significantly higher in children not infected with RVA (37.7%) compared to children with RVA positive rapid test (16.5%) (p<0.0001), mainly considering the group age under 12 months (p = 0.006) (Table 2). Stunting continued to be significantly associated with the absence of RVA infection (OR adj = 2.7, 95% CI: 1.390–5.158), considering age, gender and infection by enteric pathogens other than RVA, when a multiple logistic regression model was applied (Hosmer and Lemeshow test, p = 0.681).

Clinical features associated with RVA infection and hospitalization

With the exception of vomiting, that was significantly associated with RVA infection (p<0.001), the percentage of children exhibiting AGE related symptoms (fever and duration of diarrhea in days) and signs (lethargy, depressed fontanelle, sunken eyes and loss of skin

Table 3. Clinical features and hospitalization of children with diarrhea attended at the Bengo General Hospital.

		RV (+)	RV (-)	*p-value*
		n (%)	n (%)	
Signs and symptoms	**Vomiting** (N = 342)	N = 86	N = 256	
	Yes (N = 175)	62 (72.1)	113 (44.1)	**<0.001***
	No (N = 167)	24 (27.9)	143 (55.9)	
	Fever (N = 342)	N = 86	N = 256	
	Yes (N = 255)	69 (80.2)	186 (72.7)	0.163
	No (N = 87)	17 (19.8)	70 (27.3)	
	Duration of diarrhea in days (N = 333)	N = 85	N = 248	
	1–3 days (N = 250)	68 (80.0)	182 (73.4)	0.432
	4–5 days (N = 53)	10 (11.8)	43 (17.3)	
	≥ **6 days** (N = 30)	7 (8.2)	23 (9.3)	
	Lethargy (N = 325)	N = 86	N = 239	
	Yes (N = 93)	29 (33.7)	64 (26.8)	0.222
	No (N = 232)	57 (66.3)	175 (73.2)	
	Depressed fontanelle (N = 320)	N = 86	N = 234	
	Yes (N = 48)	14 (16.3)	34 (14.5)	0.698
	No (N = 272)	72 (83.7)	200 (85.5)	
	Sunken eyes (N = 329)	N = 85	N = 244	
	Yes (N = 76)	22 (25.9)	54 (22.1)	0.480
	No (N = 253)	63 (74.1)	190 (77.9)	
	Skin (lost of elasticity) (N = 318)	N = 86	N = 232	
	Yes (N = 13)	5 (5.8)	8 (3.4)	0.349
	No (N = 305)	81 (94.2)	224 (96.6)	
Origin and referral of the patient	**Admission service** (N = 342)	N = 86	N = 256	
	Outpatient department (N = 164)	29 (33.7)	135 (52.7)	**0.002****
	Emergency unit (N = 178)	57 (66.3)	121 (47.3)	
	Hospitalization (N = 302)	N = 74	N = 228	
	Yes (N = 81)	24 (32.4)	57 (25.0)	0.210
	No (N = 221)	50 (67.6)	171 (75.0)	

*OR = 3.3; 95%CI] 1.921–5.564[

**OR = 0.46; 95%CI] 0.274–0.759[

https://doi.org/10.1371/journal.pone.0176046.t003

elasticity) was similar in children with or without RVA infection (Table 3). Furthermore, from data available on hospitalization regarding 302 children with AGE, it was observed that 81 (26.8%) were hospitalized, of which 24 (29.6%) were RVA positive cases (Table 3). Hospitalization was non-significantly more common in RVA-positive cases than in RVA-negative cases (32.4% versus 25.0%). Regarding the admission service, there was an excess of RVA positive children at the emergency unit, which proved to be highly significant (p = 0.002) (Table 3).

Seasonality of RVA infection

During the study period, there was a substantial increase in diarrhea cases attended at the BGH during the dry season, from May to October, with a peak in May 2013 (46 cases) (Fig 1). RVA infection was detected throughout the study, with higher rates of RVA infection detected in February (60.0%), March (40.0%), June (40.0%) and July (40.9%) 2013 (Fig 1).

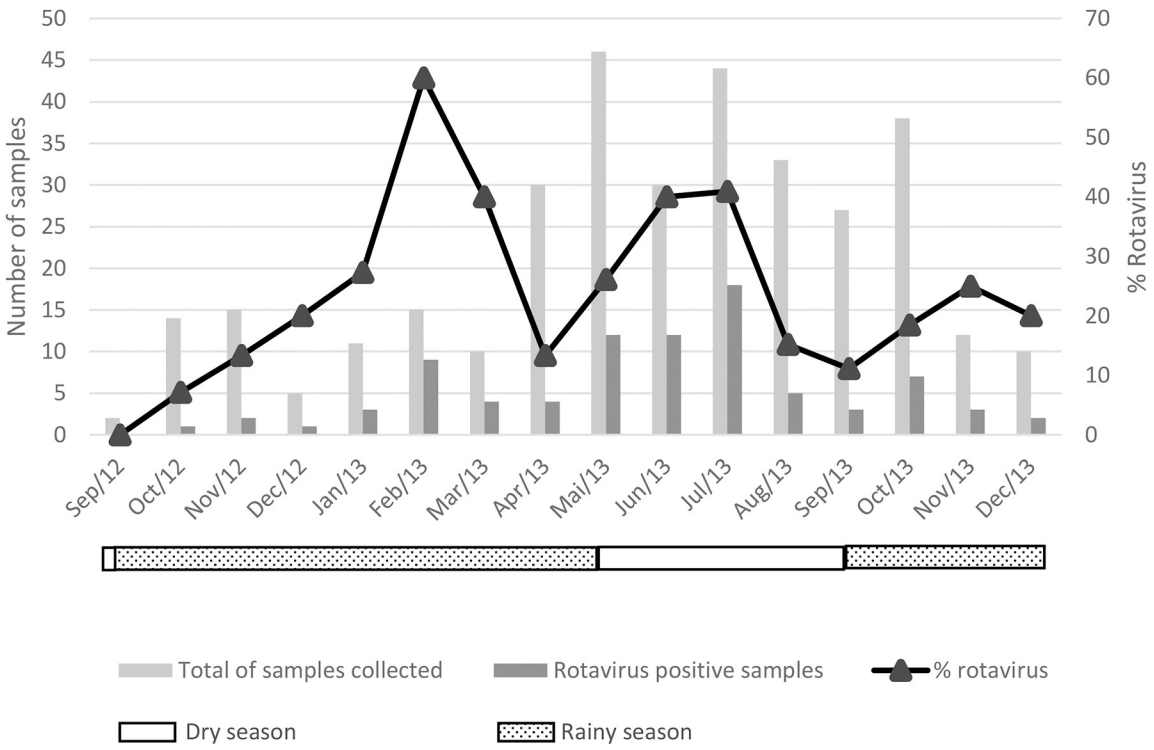

Fig 1. Seasonality of rotavirus a antigen detection in stool samples of children with diarrhea attended at the Bengo General Hospital.

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RVA genotypes: High prevalence of G1P[8] and G1P[6]

From the 86 RVA positive samples detected by rapid immunochromatographic assay, 76 (88.4%) were analyzed by molecular biology methods (the remaining 10 samples had insufficient biological material to proceed with the subsequent analysis). These RVA positive specimens (n = 76) were then further characterized for the VP6, VP4 and VP7 genes by PCR protocols using specific primers [31–34]. Of these, four samples proved to be negative by PCR. For the other 72 samples, the G and P genotypes were determined and results are presented in Fig 2.

The most prevalent genotype combination was G1P[8] (47.2%, 34/72), followed by the unusual G1P[6] (29.2%, 21/72) and G2P[4] (12.5%, 9/72). Only two G-types were found, G1 (83.3%, 60/72) and G2 (15.3%, 11/72), and for one sample it was not possible to determine the G-type. Among the P-genotypes, P[8] was the most prevalent (47.2%, 34/72), followed by P[6] (30.6%, 22/72) and P[4] (12.5%, 9/72). The determination of the P-type was not possible for 7 samples (9.7%, 7/72). When VP6 subgrouping was considered, it was observed that 84.7% (61/72) of the specimens belong to SGII and only 15.3% (11/72) were assigned to SGI. The more frequently detected genotypes G1P[8] and G1P[6] were all assigned to SGII, while G2P[4] to SGI.

Phylogenetic analysis of VP4 and VP7 genes

Randomly selected PCR amplicons from each identified genotype and from non-typable strains were sent for sequencing. Phylogenetic trees were built with partial reference sequences of VP4 and VP7 genes and are presented in Fig 3A and 3B. The RVA sequences obtained in this study clustered together in well-defined monophyletic groups with reference sequences, with significant bootstrap values (≥92%), defining very robust VP4 and VP7 genotypes.

The Angolan VP4 sequences (n = 17) were assigned to P[4] genotype lineage V (n = 2), P[6] genotype lineage Ia (n = 12) and P[8] genotype lineage III (n = 3) (Fig 3A). The P[4] strains tightly clustered with sequences of African origin, namely from Angola (KT225674, KT225677) and Burkina Faso (JX154455). The P[6] sequences clustered with sequences from Angola (KT225696, KT225681, KT225711) and other sub-Saharan African countries, such as São Tomé and Príncipe (STP) (KF356342) and Burkina Faso (JN116516), as well as with other reference sequences from Brazil (JQ693567) and the USA (JF460815). P[8] sequences, besides with Angolan sequences (KT225720, KT225724, KT225723), and from other African countries, such as STP (KF356333), Malawi (AJ302147) and South Africa (KF636182), clustered with internationally well characterized strains from USA (HM773747), Belgium (HQ392119) and Bangladesh (DQ146652). The P [8] sequences from Bengo clustered all together in the same lineage (3), different from the lineages of the two vaccines Rotarix (lineage 1) and Rotateq (lineage 2).

The Angolan VP7 sequences (n = 14) were assigned to only two genotypes, G1 (lineage I) (n = 11) and G2 (lineage IIa) (n = 3) (Fig 3B). The G1 (lineage I) monophyletic group included the Bengo sequences under study and other sequences from Angola (KT225660, KT225654, KT225650, KT225657, KT225658) and from other African countries, like STP (KF356355), the Democratic Republic of the Congo (KC510182) and South Africa (KF636184). This lineage (I) does not include the two vaccine strains Rotarix (assigned to lineage II) and Rotateq (assigned to lineage III). The three sequences classified as G2 were clustered in lineage IIa with other sequences from Angola (KT225664, KT225661) and Burkina Faso (JX154537).

Discussion

Although licensed since 2006, rotavirus group A (RVA) vaccines started to be introduced with GAVI support in African countries only since the end of 2011, Sudan being the first designated country. Presently, as many sub-Saharan African countries are still introducing the RVA vaccine in their Expanded Programme on Immunization (EPI), epidemiological studies on RVA burden and disease are still highly important.

In Angola, a leading country in diarrheal diseases, the first studies on this issue were initiated in 2012 in four provinces [22] but neither contemplating Bengo province, nor a year-

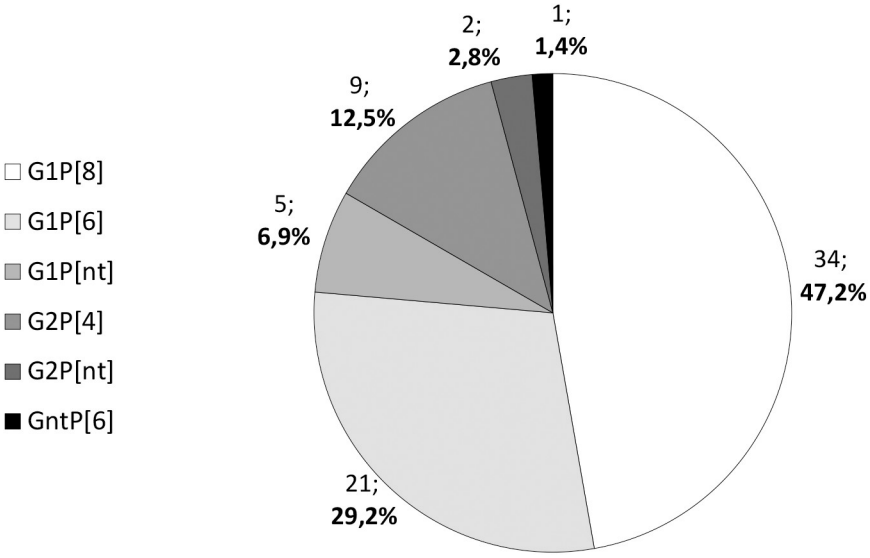

Fig 2. Distribution of G and P genotypes of rotavirus a strains in children with diarrhea attended at the Bengo General Hospital.

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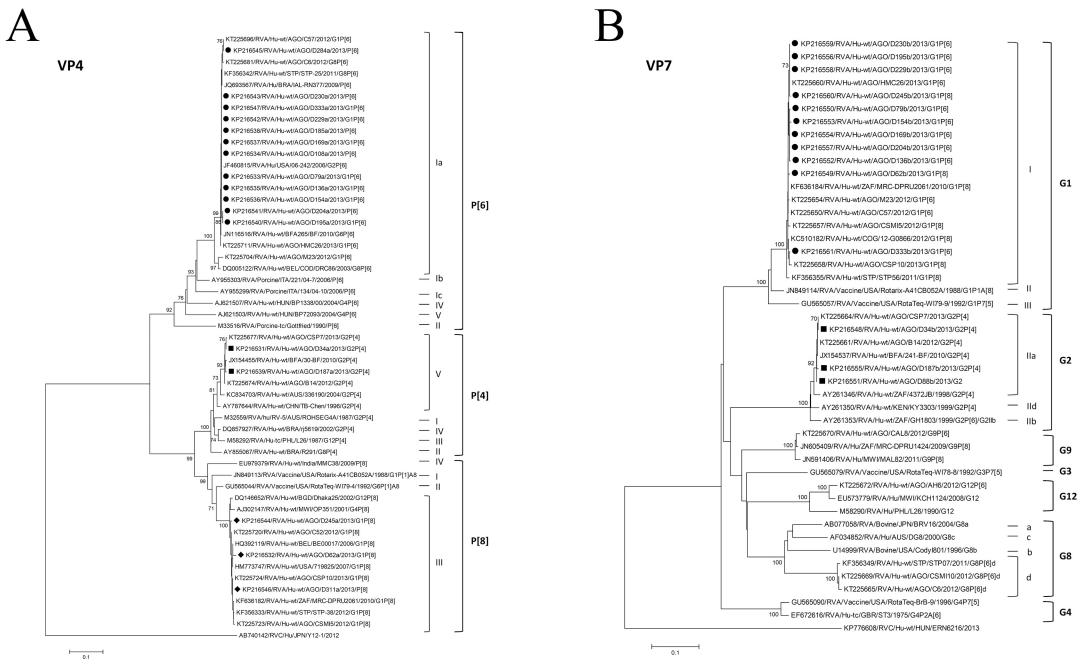

Fig 3. Phylogenetic analysis of the VP4 (A) and VP7 (B) genes from RVA identified strains. (A) Genotypes P[6] (closed circles), P[4] (closed squares), and P[8] (closed diamonds) (B) G1 (closed circles) and G2 (closed squares) detected in Bengo, Northwestern Angola. The analysis involved, respectively for VP4 and VP7, 53 and 46 nucleotide sequences and a total of 468 and 627 positions in the final datasets. Phylogenetic analysis was performed using a distance-based neighbour-joining method, based on the Kimura 2-parameter model, with 1,000 bootstrap resamplings (percent bootstrap values of more than 70 are indicated at each node). The tree is drawn to scale, with branch lengths proportional to the evolutionary distances.

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round sampling. To overcome this, between September 2012 and December 2013, a cross-sectional study was conducted at the Bengo General Hospital (BGH) in Caxito, Bengo province, Angola. RVA detection rate (25.1%) was considered within the range of other African countries [16] although lower than the total detection rate reported for other provinces of the country (35%) [22]. The AdV detection rate was much lower (3.8%), but similar to the rates registered in other sub-Saharan African countries such as Tanzania [38] and the Republic of the Congo [39] before RVA vaccine introduction.

In addition to RVA infection rate (and genotype distribution), we have also investigated several socio-demographic characteristics of the target population trying to get a better insight of the general epidemiology of this virus within this population in Angola.

As shown in most of the African studies including neighboring countries such as Botswana [40] or the Republic of the Congo [39] or considering RVA epidemiology in other provinces of Angola [22], the group of children more significantly affected by RVA AGE was below 12 months of age. This shows a shift of RVA infection towards earlier age in most African settings studied so far, the age factor being considered determinant for vaccination schedule set up, further showing the importance of neonatal vaccine administration in African countries in order to prevent RVA disease [41].

Considering earlier reports which associated maternal literacy and an increased level of education with protection against child mortality and diarrheal diseases [42, 43], we looked into these socio-demographic characteristics, but similarly to Mozambique [44], no correlation was found. Although some studies [45] point towards a correlation between the level of

maternal education and some markers of child health, a causal relationship is not unequivocally established.

The high burden of diarrheal diseases, especially affecting developing countries, was previously associated with limited access to drinking water and poor sanitation conditions, factors that facilitate transmission of enteric pathogens [46] thus of RVA. Considering the Global Enteric Multi-Center Study (GEMS) from 2012, in sub-Saharan Africa, 83% of urban and 49% of rural populations have access to improved water sources, while the numbers are much lower when access to improved sanitation facilities is considered (43% of urban and 23% of rural populations) [46]. Improving these conditions became a strong priority and one of the Millennium Development Goals of the WHO. In this study, 28.7% (98/342) of the inquired mothers/caretakers declared to use water from the local Central Water Treatment Plant (CWTP) exactly as supplied, while 59.9% (205/342) applied a domestic treatment, including the use of bleach or alum stone, boiling and/or filtration, to the water provided for the household, irrespective of the source (the CWTP or other sources. i.e., directly from rivers, irrigation channel or boreholes). However, the procedure of home-treatment of drinking water is very difficult to verify (almost impossible to check for the boiling time, if using the correct bleach concentration or type of filters, how to combine with alum stone, etc.) and presumably not always correctly implemented. That is one of the reasons why the results obtained should be taken very cautiously. It is noteworthy that 11.4% (39/342) of the children drank untreated water. As stated before, none of the categories considered seemed to be significantly associated with RVA infection in this study. Comparing proportions of RVA-positive and -negative children of different age groups, no significant association emerged concerning the drinking water source and also the treatment method applied. The lowest RVA detection rate of 20.5% (8/39) was obtained in the group using water originating from alternative sources to the CWTP, with no treatment, and the highest RVA detection rate of 27.6% (27/98) in the group using water from the CWTP with no additional home treatment. Although at first this could mean that children drinking water from CWTP were the most exposed to RVA infection, our statistical analysis does not seem to firmly support that statement.

Despite the absence of any significant differences between the rates of RVA infection by the type of sanitation facilities reported (Table 2), on the overall and when considering each age group, it was interesting to observe that RVA detection rate is the lowest in the absence of latrines (20%, 14/70) and the highest with the use of public latrines (27.8%, 35/126), irrespective of the presence of running water. This could be explained by the fact that latrine sharing creates unsanitary conditions (and keeping the community toilet clean could be difficult under the circumstances), thus creating conditions for a more widespread fecal-oral transmission of enteric pathogens and in this case RVA. To overcome this, proper cleaning and disinfection, implementation of hygienic measures as well as healthy sanitation behavior should be promoted and implemented at household level and particularly at improved public sanitation facilities (use of latrines with running water).

Although no significant association of RVA infection and the drinking water source, water treatment or furthermore hygiene practices related to sanitation facilities of the family inquired was found, we would like to highlight the importance of implementing appropriate and regular "home" treatment of the drinking water, adequate storage and pipeline distribution for the water of the CWTP. We cannot disregard the putative impact of the current poor sanitation conditions on the transmission of other enteric pathogens (not addressed in this study).

The interference of maternal antibodies in breast milk, one of the putative causes that has been put forward to explain RVA vaccine lower efficacy in developing countries, was studied in sub-Saharan settings before [44] and after the vaccine introduction [47]. Recent post-

vaccination studies in Zambia and South Africa suggested that lower immunogenicity of Rotarix vaccine could be explained partially by exposure to high antibody titres in breast milk and early exposure to wild-type rotavirus infections [47, 48]. Some pre-vaccination studies from developed [49] as well developing countries [50] indicated that breastfeeding protects infants against AGE caused by RVA. However, our results confirmed the hypothesis presented by a recent study from Mozambique [44] in which no significant protective effect against RVA infection could be associated with breastfeeding.

Rotavirus infection and malnutrition are common in children in the developing countries, thus we investigated the nutritional status of children enrolled. A recent study in Bangladesh showed that a better nutritional status is positively associated with RVA diarrhea in the first three years of life [51]. However, in earlier studies from Africa, wasting was associated with more severe forms of diarrhea and RVA infection [52], showing that the impact of nutritional status on susceptibility to RVA diarrhea is a controversial subject, far from being completely understood. In our study, signs of malnutrition were quite evident, in a high proportion of the children enrolled. Although underweight and wasted children seemed more prone for RVA infection, no significant association was established. On the other hand, stunting (particularly in children below 12 months of age) was significantly more common in children not infected with RVA. Despite the differences observed, we cannot exclude the influence of other factors affecting nutritional status such as maternal stunting and birth weight that were not considered in our study.

In terms of clinical features potentially associated with RVA infection, only vomiting was highly significantly associated with RVA infection. This association was also shown before in other sub-Saharan African countries [52]. As frequent as it may seem in RVA-infected children, clinical management of vomiting requires a special attention, since prolonged vomiting in these small, undernourished, children may rapidly deplete the body of water (dehydration), profoundly impacting the electrolyte equilibrium.

Hospitalization for diarrheal diseases and specifically for RVA infection has been associated with a significant burden to the health systems and households in developing countries [53]. In our study, we observed that the percentage of hospitalization in children with RVA infection (32.4%) was higher than in RVA negative children (25.0%), and that there was a highly significant excess of RVA positive children at the emergency unit, when considering the admission type for the AGE cases at the BGH. Before RVA vaccine introduction, studies to evaluate its economic impact were undertaken. The great benefit of RVA vaccination, e.g. for developing, GAVI-eligible countries, has been shown [54] namely in the reduction of the burden of disease in already fragile healthcare facilities.

Although several studies from sub-Saharan African countries point for a higher prevalence of RVA infection in the dry season [20, 39, 55], no seasonality of RVA infection was evident in our year-round study. Nevertheless, an apparent increase in diarrhea cases admitted at the BGH during the dry season seems plausible (Fig 1), showing the possibility of other enteric agents being involved as etiologic agents.

Currently, a great regional and temporal variety of RVA strains have been reported worldwide [21]. Their relative importance before and after RVA vaccine introduction, especially in developing countries, where concerns aroused about strain replacement by emergence of novel strains which could lower vaccine efficacy, has been acknowledged. Five viral strains were reported as most prevalent globally before RVA vaccine introduction in the national immunization programmes, the most prevalent being G1P[8], identified as dominant also in the scope of this study (47.2%, 34/72). On the overall, this strain is considered as responsible for more than 50% of RVA infections in children and is part of both licensed vaccines (Rotarix

and Rotateq), but considering AFROROTANET data a lower detection rate (14–31%) was observed in African countries [16].

Another identified genotype, G1P[6], the second most detected in our study (29.2%, 21/72) has been shown as an emerging strain in recent years. Considering the period 2007–2012, after the RVA vaccines being licensed globally but before introduction in most of African countries, P[6]-type were increasingly detected in Africa, with G1P[6] detection rate established as 6% [21]. After RVA vaccines introduction in African countries, this strain became one of the six most prevalent strains throughout the continent [56], fact that our results seem to corroborate.

Finally, G2P[4], a strain considered predominant as before as after RVA vaccine introduction, registered a detection rate of 12.5% (9/72) in our study. This detection rate is comparable with data reported globally before vaccine introduction (10.6%) [15] but also afterwards (13%, between 2007–2012) [21], as well as with reports from African countries during the period 2007–2013 (10.5%) [56].

The G and P-types identified in this study clustered together respectively with other G1 and G2, and P[4], P[6] and P[8] strains, from sub-Saharan African countries and also international references, sharing a high nucleotide identity with strains detected previously in other provinces of Angola [22]. Albeit the long geographic distances involved, when comparing sampling sites in the two studies, there is a remarkable genetic homogeneity within each genotype, both for VP4 and for VP7 (Fig 3A and 3B). The limited number of genotypes found in this study probably reflects a limited number of recent introductions in the study area of Bengo province in Angola. The identified sequences were assigned to genotypes and furthermore to lineages (e.g. GI-I and P[8]-III) although these lineages are different of the ones included in currently used RVA vaccines (G1-II for Rotarix and G1-III for RotaTeq, P[8]-I for Rotarix and P[8] -II for RotaTeq).

Although P[6] was initially considered of zoonotic origin [18] and assigned to SGI [18, 57], the combination G1P[6] was shown to belong to SGII, as also shown before in the previous Angolan study [22], and in the same subgroup as the well-studied G1P[8].

Finally, in Africa, P[6] RVA infection, described as emerging, has been associated with host genetic factors (e.g. Lewis phenotype), apparently infecting more frequently Lewis-negative children [58]. This Lewis phenotype was also shown to be more common in many African populations than in Caucasian populations of Europe and North America, and thus this fact could explain the increase of this strain detection rate in Africa. Further host genetics studies could elucidate the P[6] association to Lewis-negative phenotype and get an insight on future vaccine efficacy in this population, known the lower efficacy of RVA vaccines in low and middle-income African populations.

Conclusions

In this hospital-based study, aiming to provide baseline information before vaccine introduction in Angola, the detection rate of RVA infection and genotype distribution in the Bengo province have been investigated. A high detection rate was registered (25.1%) and the most prevalent genotypes were shown to be G1P[8] (47.2%), followed by G1P[6] (29.2%) and the less detected G2P[4] (12.5%). The natural variability of RVA strains in Africa was demonstrated by many studies including the present one, stressing the fact that epidemiological surveillance prior and post RVA vaccine introduction would be crucial to detect potential emergence of new genotypes. Apart from age and age distribution, there was no statistically significant association between RVA infection and any other socio-demographic variable analyzed. Regarding clinical features associated with RVA infection, only vomiting proved to be significantly associated with RVA infection. Although there was an increase in diarrhea cases

registered at the BGH during the dry season, no seasonality of RVA infection was proved during the study period.

Limitations

- Reduced amount of collected sample thus not allowing to genotype all RVA positives detected by immunochromatographic rapid test.
- According to the inclusion criteria set up, children presenting with vomiting only were not enrolled in this study, which may impact on disease burden, underestimating the RVA rate of infection.

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ORIGINAL ARTICLE

Molecular characterization of *Giardia lamblia* in children less than 5 years of age with diarrhoea attending the Bengo General Hospital, Angola

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Background: *Giardia lamblia* is a pathogenic intestinal protozoan with high prevalence in developing countries, especially among children. Molecular characterization has revealed the existence of eight assemblages, with A and B being more commonly described in human infections. Despite its importance, to our knowledge this is the first published molecular analysis of *G. lamblia* assemblages in Angola.

Methods: The present study aimed to identify the assemblages of *G. lamblia* in children with acute diarrhoea presenting at the Bengo General Hospital, Angola. A stool sample was collected and microscopy and immunochromatographic tests were used. DNA was extracted and assemblage determination was performed through amplification of the gene fragment *ssu-rRNA* (175 bp) and *β-giardin* (511 bp) through polymerase chain reaction and DNA sequencing.

Results: Of the 16 stool samples screened, 12 were successfully sequenced. Eleven isolates were assigned to assemblage B and one to assemblage A. Subassemblage determination was not possible for assemblage B, while the single isolate assigned to assemblage A was identified as belonging to subassemblage A3.

Conclusion: This study provides information about *G. lamblia* assemblages in Bengo Province, Angola and may contribute as a first step in understanding the molecular epidemiology of this protozoan in the country.

GenBank accession numbers for the *ssur-RNA* gene: MF479750, MF479751, MF479752, MF479753, MF479754, MF479755, MF479756, MF479757, MF479758, MF479759, MF479760, MF479761.
GenBank accession numbers for the *β-giardin* gene: MF565378, MF565379, MF565380, MF565381.

Keywords: Angola, Children, Diarrhoea, Genotyping, *Giardia lamblia*, Hospital

Introduction

Giardia lamblia is a common intestinal parasite infecting a broad range of vertebrate species, including humans.[1] This parasite has a global distribution and it is estimated that 280 million people are infected worldwide,[2,3] with 200 million people presenting symptomatic giardiasis in the developing countries.[2,4] Children living in developing countries with poor hygiene and

sanitation conditions[5] are more vulnerable to the clinical consequences of *G. lamblia* infection.[6] The harmful effect of giardiasis on growth and development in children has been observed in several studies and the potential effects of chronic malnutrition on cognition, intelligence and psychomotor development have also been described.[7,8] Several studies have shown that malnutrition in children can be an important consequence of *Giardia* infection.[9–12] However, there are few published studies in recent years exploring the association of the assemblage of *G. lamblia* with infant nutritional status.[12,13]

Currently *G. lamblia* is considered a species complex, comprising eight assemblages (A–H).[6] The majority of these assemblages are host specific, but only assemblages A and B are known to infect humans,[14,15] with assemblage B being described as more common.[3] A recent study reported for the first time assemblage E in humans in Australia.[16] However, the number of molecular epidemiological studies of giardiasis in humans is not enough to determine geographic or socio-economic differences in the distribution of assemblages A and B.[6]

The severity of the disease is determined by the interaction among parasite's virulence, host's nutritional and immunological status, nature of the intestinal microflora and the presence or absence of other pathogenic intestinal agents.[6] Although the different assemblages of *G. lamblia* may eventually produce different toxins or metabolic products that contribute to their pathogenicity[17] or differences in antigenic variation and host specificity,[18–20] studies on the possible association between the genetic groups of *G. lamblia* and their virulence (defined by the probability of causing diarrhoea and other clinical symptoms) continue to show inconsistent results.[6,21,22] Although several studies have shown a correlation between *G. lamblia* infections belonging to assemblage B with more severe symptoms,[23–26] others have correlated the most severe symptoms with assemblage A.[20,22]

An interesting observation raised by Almeida et al.[21] was that there seems to be a relationship between the molecular marker used in the study and the assemblage believed to be more aggressive. These investigators found that almost all genotyping studies based on the *ssu-rRNA* and *tpi* genes support the idea that assemblage A is associated with symptomatic disease, whereas studies based on the *bg* and *gdh* genes associated the symptomatology with assemblage B.[21]

The occurrence and spread of giardiasis in human populations is an emerging public health problem around the world. Molecular studies are essential to clarify the importance of local *Giardia* assemblages.[27]

In Angola, there is limited information available concerning infection with intestinal parasites and only in recent years have some studies been published.[28–31] Two of these studies report *G. lamblia* as being the most prevalent intestinal protozoan in children from the province of Bié and the city of Lubango (Huíla province).[28,31] We have no knowledge of previous studies on molecular characterization of *G. lamblia* carried out in Angola.

The aim of the present study was to perform a genetic characterization of *G. lamblia*, using polymerase chain reaction (PCR) and DNA sequencing, in children with diarrhoea attending the Bengo General Hospital.

Materials and methods

Population and study design

This study is part of a cross-sectional study conducted between September 2012 and December 2013 at the Bengo General Hospital, located in Caxito, the capital city of Bengo Province, 60 km northeast of Luanda, Angola.[32] The study aimed to investigate the aetiology of diarrhoea in children younger than 5 y attending the paediatric emergency service or the hospital outpatient paediatric unit with diarrhoea (three or more loose or liquid stools per day).[32] Children receiving antibiotics or antiparasitic drugs were excluded in order to avoid false negatives. A survey including sociodemographic characterization, information on breastfeeding practices, water source and sanitation conditions was applied by the clinical staff. Symptomatology in the previous 10 days (diarrhoea, vomiting, fever and bloody diarrhoea) was reported by caregivers. Anthropometric measurements (weight and length/height) were assessed to calculate anthropometric indices expressed as a Z-score for each child.[33]

The study protocol was approved by the Ethics and Committee of the Angolan Ministry of Health and the Ethics Committee of the Instituto de Higiene e Medicina Tropical, Portugal. Written informed consent and voluntary consent was obtained from parents or legal guardians prior to the inclusion of each child.

Stool sample collection and initial diagnosis of *G. lamblia*

A single stool sample per child was collected to a sterile container provided by the clinic staff. Once delivered to the laboratory, stool samples were immediately processed for microscopic identification of cysts and/or trophozoites of *G. lamblia* (direct examination with saline and iodine and a concentration method [ParasiTrap system, Biosepar, Mühldorf, Germany]) followed by the detection of *Giardia* antigen through immunochromatographic rapid tests (RIDAQUICK *Cryptosporidium/Giardia* Combi, R-Biopharm, Darmstadt, Germany). Microscopy and immunochromatographic rapid tests were performed in 344 and 338 children, respectively. Positive samples by at least one of the methods applied were preserved at −20°C for DNA extraction (Figure 1).

DNA extraction

DNA was extracted from *G. lamblia*–positive stool samples preserved at −20°C using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, except for the final step where 100 µl of the elution solution were used.

DNA amplification

The DNA of the extracted samples was amplified through PCR, using primers targeting the small subunit ribosomal RNA (ssu-rRNA)[34,35] (Figure 1). The primers used were RH11: 5′-CAT CCG GTC GAT CCT GCC-5′ and RH4: 5′-AGT CGA ACC CTG ATT CTC CGC CCA GG-3′ for the first reaction and GiarF: 5′-GAC GCT CTC CCC AAG GAC-3′ and GiarR: 5′-CTG CGT CAC GCT GCT CG-3′ for the

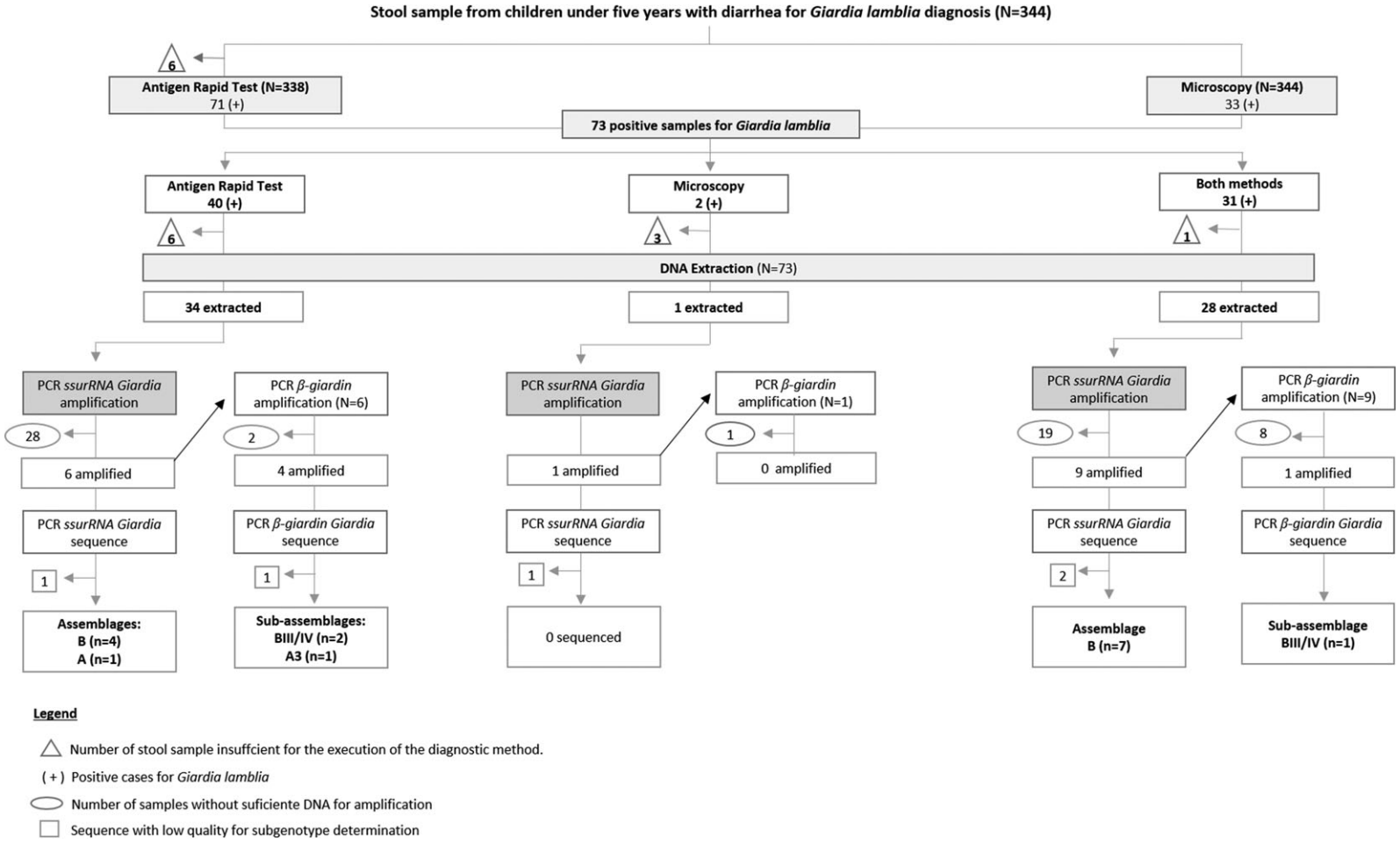

Figure 1. Molecular characterization of *Giardia lamblia* detected by microscopy and/or rapid antigen test in stool samples from children with diarrhoea attending the Bengo General Hospital, Angola.

secondary PCR.[34,35] All samples successfully amplified for ssu-rRNA were posteriorly amplified for the β-giardin (bg) loci (Figure 1). The primers used were G7: 5′-AAG CCC GAC GAC CTC ACC CGC AGT GC-3′ and G759: 5′G AG GCC GCC CTG GAT CTT CGA GAC GAC-3′ for the first reaction and G8: 5′-GAA CGA ACG AGA TCG AGG TCC G-3′ and G9: 5′-CTC GAC GAG CTT CGT GTT-3′.[36,37]

Amplification reactions were performed using 2 µl of DNA template in a final volume of 25 µl, using illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Little Chalfont, UK). Both positive (DNA isolated from the Portland-1 strain; ATCC 30888D, ATCC-LGC Promochem, Manassas, VA, USA) and negative controls (no template added) were included in each series of PCRs. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

DNA sequence analysis

For sequence analysis, PCR products of amplified samples were purified using illustra GFX PCR DNA and the Gel Band Purification Kit (GE HealthCare) according to the manufacturer's instructions. DNA sequencing reactions were carried out in both directions for ssu-rRNA (175 bp) and β-giardin (511 bp) PCR-generated fragments.

Sequences obtained (75.0% [12/16] for ssu-rRNA and 80.0% [4/5] for β-giardin) were aligned with previously published sequences of *G. lamblia* isolates available in the GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), using BLAST for assemblage determination and Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) for subassemblage determination.

Results

Microscopic identification and antigen detection of *G. lamblia* was performed in a total of 338 children, of whom 73 (21.6%) were positive: 40 (54.8%) were only detected through immunochromatographic tests, 2 (2.7%) only by microscopy and 31 (41.2%) through both methods (Figure 1). DNA extraction was performed in 63 positive samples, of which 16 were amplified for the *ssu-rRNA* gene fragment and 12 were successfully sequenced (Figure 1). All analysed samples belonged to assemblage B (11/12), except one that belonged to assemblage A (1/12) (GenBank accession numbers: MF479750, MF479751, MF479752, MF479753, MF479754, MF479755, MF479756, MF479757, MF479758, MF479759, MF479760, MF479761) (Table 1). These samples were also amplified for the *bg* gene fragment, for subassemblage determination, but only four sequences had enough quality for sequencing, three belonging to assemblage B and one to assemblage A (GenBank accession numbers: MF565378, MF565379, MF565380, MF565381) (Table 1). It was not possible to determine the subassemblages belonging to assemblage B due to the high polymorphism observed in the chromatogram. However, the isolate belonging to assemblage A was found to belong to the A3 subassemblage (Table 1).

Discussion

To our knowledge this is the first study on the molecular characterization of *G. lamblia* in Angola. Despite the small number of the samples studied, in the present study assemblage B was clearly predominant (93.8%), with only one sample assigned to assemblage A. The prevalences of assemblages A and B are differently distributed among and within countries, although there is no solid evidence that explains this geographic variation around the world.[22] Studies conducted in Mexico, Brazil and Colombia have identified high frequencies of assemblage A, while studies in Nicaragua and Argentina have shown that assemblage B predominates in these countries.[38–40] In South and Southeast Asia, including India, assemblage A seems to predominate, just as in Europe.[41–43] Reports from Africa have also shown high prevalences of assemblage B in countries such as Egypt (80%), Rwanda (85.9%) and Morocco (81.8%).[12,44,45]

It has been suggested that assemblage A is associated with zoonotic transmission, since it is commonly found in a wide range of animals, including in a large number of cattle.[6,37,46] Assemblage B was associated with a higher rate of cyst shedding compared with assemblage A in a study conducted in Brazilian children, which may contribute to a greater spread and consequently a higher incidence of assemblage B.[47] In this study, assemblage B was more common in the studied children and this could indicate that the major transmission route was more likely to occur between humans. Indeed, in the studied population about 20% of the households did not have a latrine, which may contribute to *G. lamblia* faecal–oral transmission between humans.[32] Furthermore, there are no clear geographic differences or socio-economic factors responsible for the distribution of both assemblages.[6,37,48]

Assemblage B is considered genetically diverse and the isolates present a high substitution rate that makes the real subassemblage patterns difficult to determine.[49] The difficulty in defining subassemblages in assemblage B is described in several studies.[3,39,50,51]

This study has some limitations. This is a hospital-based study that included children less than 5 years of age with diarrhoea, thus the results on the prevalence of *G. lamblia* and respective assemblages cannot be generalized to the entire population. To overcome this limitation, since infection by this protozoan is mainly asymptomatic,[52,53] a case–control design would be interesting in order to compare the prevalence of *G. lamblia* and its assemblages with other enteric pathogens in participants with diarrheal and non-diarrheal stools. Moreover, in diarrheal samples the non-resistant forms of this pathogen (trophozoites) are predominant, which can impair the microscopic diagnosis. The use of immunochromatographic tests was able to prevent this problem. It was not possible to assemblage some stool samples, probably due to freezing and thawing of the samples since their initial collection, which may contribute to DNA degradation. The limited number of samples studied, with only one assemblage A being detected, precludes investigating the differences between assemblage A and B in relation to symptomatology, malnutrition and sociodemographic conditions.

Despite the limitations mentioned above, we believe that an important contribution was made through the identification of *G. lamblia* assemblages circulating in children attending the Bengo General Hospital, Angola.

Future investigations are needed to clarify the current genetic diversity and better understand the importance of local *G. lamblia* assemblages. Studies with a larger number of samples, also including information about the seasonality of *G.*

Table 1. *Giardia lamblia* assemblages identified in children with diarrhoea attending the Bengo General Hospital, Angola (N=12) and respective descriptions of sample collection (month/year), gender (male/female), age in months, reported symptomatology (vomiting, fever and lethargy), type of enteric infection identified according to the enteric pathogens detected (simple: the child was infected only by *G. lamblia*; multiple: the child was infected by *G. lamblia* and other enteric pathogens), laboratory results of microscopy and rapid test detection methods performed.

Identification		Sociodemographic characterization		Symptomatology			Enteric infection	Diagnosis of *Giardia lamblia*					
Number	Collection date (month, year)	Gender	Age (months)	Vomiting	Fever	Lethargy	Type	Microscopy	Rapid test	Molecular assemblages			
										ssu	GenBank accession nos.	bg	GenBank accession nos.
1	February 2013	Male	11.4	Yes	Yes	No	Multiple	Negative	Positive	B	MF479750	n.a.	
2	April 2013	Female	10.5	Yes	Yes	Yes	Simple	Negative	Positive	A	MF479751	A3	MF565378
3	May 2013	Female	8.6	No	Yes	Yes	Multiple	Negative	Positive	B	MF479752	B*	MF565379
4	May 2013	Male	11.3	Yes	Yes	No	Multiple	Negative	Positive	B	MF479753	B*	MF565380
5	May 2013	Female	13.1	No	Yes	No	Multiple	Negative	Positive	B	MF479754	n.a.	
6	June 2013	Female	12.8	No	Yes	Yes	Multiple	Positive	Positive	B	MF479755	n.a.	
7	August 2013	Male	9.3	No	Yes	No	Multiple	Positive	Positive	B	MF479756	n.a.	
8	August 2013	Female	27.4	No	No	No	Simple	Positive	Positive	B	MF479757	B*	MF565381
9	August 2013	Female	14.2	No	No	No	Multiple	Positive	Positive	B	MF479758	n.a.	
10	August 2013	Male	27.3	Yes	Yes	No	Multiple	Positive	Positive	B	MF479759	n.a.	
11	October 2013	Female	30.9	No	Yes	Yes	Simple	Positive	Positive	B	MF479760	n.a.	
12	December 2013	Male	17.3	No	Yes	No	Multiple	Positive	Positive	B	MF479761	n.a.	

*Subassemblage determination not possible.
n.a.: not applicable.

lamblia prevalence and the zoonotic potential of this protozoan, would aid our understanding of the epidemiology of giardiasis in Angola, contributing to better definition of key priorities in its control.

Authors' contributions: CG participated throughout the entire sampling, carried out parasitological analysis (microscopy and immunochromatographic rapid tests), contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. FSF carried out molecular analysis, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. ACM coordinated and supervised the clinical staff, critically reviewed the manuscript and approved the final manuscript as submitted. MCM carried out parasitological analysis (microscopy and immunochromatographic rapid tests), drafted the initial manuscript, critically reviewed the manuscript and approved the final manuscript as submitted. SVN conceptualized and designed the study, coordinated the planning phase of the study, contributed to the analysis and interpretation of data, critically reviewed the manuscript and approved the final manuscript as submitted. ASR carried out parasitological analysis (microscopy and immunochromatographic rapid tests) and DNA extraction, contributed to both data analysis and interpretation, drafted the initial manuscript, critically reviewed the manuscript and approved the final submitted manuscript. DPC carried out molecular analysis, critically reviewed the manuscript and approved the final submitted manuscript. MB conceptualized and designed the study, coordinated and supervised data collection, contributed to the analysis and interpretation of data, drafted the initial manuscript and critically reviewed and approved the final submitted manuscript.

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Competing interests: None declared.

Ethical approval: The study protocol was approved by the Ethics Committee of the Angolan Ministry of Health and the Ethical Committee of the Instituto de Higiene e Medicina Tropical, Portugal.

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2.2 Paper IV. Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional status in Bengo, Angola: a four-arm randomised parallel trial

Reference:

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Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional status in Bengo, Angola: a four-arm randomised parallel trial

--Manuscript Draft--

Manuscript Number:	PONE-D-19-10988
Article Type:	Clinical Trial
Full Title:	Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional status in Bengo, Angola: a four-arm randomised parallel trial
Short Title:	Treatment of intestinal parasites and children nutritional status: a randomised parallel trial
Corresponding Author:	Luzia Gonçalves Instituto de Higiene e Medicina Tropical - Universidade Nova de Lisboa Lisboa, PORTUGAL
Keywords:	Longitudinal; deworming; intestinal parasites; Malnutrition; Height-for-age Z-score; Angola; growth
Abstract:	Background: Malnutrition and intestinal parasitic infections are a public health problem in children in Angola. A four-arm randomised parallel trial was longitudinally conducted to investigate if a single annual dose of albendazole (ALB) or a four-monthly test-and-treat (FMTT) intestinal parasites approach at individual or household levels improve nutritional outcomes of children (2-5 years old) in Bengo, Angola. Methods and Findings: Children infected with at least one pathogenic intestinal parasite n=121) were enrolled and randomly assigned (1:1:1:1) to: annual single dose of ALB at individual level (Arm 1, n=29); annual single dose of ALB at household level (Arm 2, n=31); FMTT approach at individual level (Arm 3, n=31); and FMTT approach at household level (Arm 4, n=30). Growth was assessed by height, weight, height-for-age (HAZ), weight-for-height (WHZ), weight-for-age (WAZ), and mid-upper arm circumference Z-score (MUACZ) at baseline, 4, 8, 12, 16, 20, and 24 months. Intention-to-treat analysis was done after a missing analysis. A non-parametric approach (nparLD), mixed effect models (LMM), and generalized estimating equations (GEEs) were used. At entry, children (26.6±4.86 months) were mainly infected by Giardia lamblia (57%) and Ascaris lumbricoides (26%). After two years, models provided no evidence or weak evidence of interventions on growth. Mean of HAZ, WAZ and MUACZ remained negative across arms, while WHZ registered positive means. Significant effects of intervention on height and WHZ was noted in GEE models. Children from A2, A4, and A3 were estimated to have 2.1 cm (SE=1.32), 1.4 cm (SE=1.44) and 0.3 cm (SE=1.26) more than those from A1. LMM provided similar estimates. The duration of the study and the sample size may have been insufficient to observe differences. Conclusions: Although none of strategies stood out significant effect on nutrition outcomes, findings highlight the burden of malnutrition in pre-school children. Future studies should consider multiple factors contributing to malnutrition (e.g., inadequate food, water, hygiene and sanitation, and health care services) in order to improve nutritional status of children. Trial registration: ISRCTN-72928001.
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Financial Disclosure Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research articles from *PLOS ONE* for specific examples. This statement is required for submission and **will appear in the published article** if the submission is accepted. Please make sure it is accurate. **Unfunded studies** Enter: *The author(s) received no specific funding for this work.* **Funded studies** Enter a statement with the following details: • Initials of the authors who received each award • Grant numbers awarded to each author • The full name of each funder • URL of each funder website • Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript? • **NO** - Include this sentence at the end of your statement: *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.* • **YES** - Specify the role(s) played. * typeset	This study was supported by the promoters of CISA (Camões, Institute of Cooperation and Language, Portugal; Calouste Gulbenkian Foundation (FCG); Government of Bengo Province; Angolan Ministry of Health - MINSA) and the National Malaria Control Program, Angola. C.G was awarded a PhD grant by the Foundation for Science and Technology (FCT), Portugal (SFRH/BD/91566/2012) and a grant by FCG (grant number 212008), Lisbon, Portugal. FCT also supported this study by projects UID/MAT/00006/2013, UID/Muli/04413/2013. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.
Competing Interests Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this	The authors have declared that no competing interests exist.

work—acknowledging all financial support and any other relevant financial or non-financial competing interests. This statement **will appear in the published article** if the submission is accepted. Please make sure it is accurate. View published research articles from *PLOS ONE* for specific examples. **NO authors have competing interests** Enter: *The authors have declared that no competing interests exist.* **Authors with competing interests** Enter competing interest details beginning with this statement: *I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]* * typeset	
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Additional data availability information:	

Full title: Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional status in Bengo, Angola: a four-arm randomised parallel trial

Short title: Treatment of intestinal parasites and children nutritional status: a randomised parallel trial

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Abstract

Background: Malnutrition and intestinal parasitic infections are a public health problem in children in Angola. A four-arm randomised parallel trial was longitudinally conducted to investigate if a single annual dose of albendazole (ALB) or a four-monthly test-and-treat (FMTT) intestinal parasites approach at individual or household levels improve nutritional outcomes of children (2-5 years old) in Bengo, Angola.

Methods and Findings: Children infected with at least one pathogenic intestinal parasite n=121) were enrolled and randomly assigned (1:1:1:1) to: annual single dose of ALB at individual level (Arm 1, n=29); annual single dose of ALB at household level (Arm 2, n=31); FMTT approach at individual level (Arm 3, n=31); and FMTT approach at household level (Arm 4, n=30). Growth was assessed by height, weight, height-for-age (HAZ), weight-for-height (WHZ), weight-for-age (WAZ), and mid-upper arm circumference Z-score (MUACZ) at baseline, 4, 8, 12, 16, 20, and 24 months. Intention-to-treat analysis was done after a missing analysis. A non-parametric approach (nparLD), mixed effect models (LMM), and generalized estimating equations (GEEs) were used. At entry, children (26.6±4.86 months) were mainly infected by *Giardia lamblia* (57%) and *Ascaris lumbricoides* (26%). After two years, models provided no evidence or weak evidence of interventions on growth. Mean of HAZ, WAZ and MUACZ remained negative across arms, while WHZ registered positive means. Significant effects of intervention on height and WHZ was noted in GEE models. Children from A2, A4, and A3 were estimated to have 2.1 cm (SE=1.32), 1.4 cm (SE=1.44) and 0.3 cm (SE=1.26) more than those from A1. LMM provided similar estimates. The duration of the study and the sample size may have been insufficient to observe differences.

Conclusions: Although none of strategies stood out significant effect on nutrition outcomes, findings highlight the burden of malnutrition in pre-school children. Future studies should consider multiple factors contributing to malnutrition (e.g., inadequate food, water, hygiene and sanitation, and health care services) in order to improve nutritional status of children.

Trial registration: ISRCTN-72928001.

Introduction

Malnutrition is the underlying cause of 45% of deaths in children under five years [1]. Most of these preventable deaths occur due to poor living conditions, reduced access to adequate nutrition, water, sanitation, basic health services, and treatment of infectious diseases [1]. Globally, 151 million children are stunted and 50.5 million are diagnosed with wasting [1], with Africa ranking highest in prevalence of stunting (30.3%) [1]. Reducing by 40% the number of under-five stunted children between 2010 and 2025 is included in the second Sustainable Development Goal [1, 2].

Soil-transmitted Helminth (STH) infections, caused by *Ascaris lumbricoides*, *Trichuris trichiura* and the hookworms *Necator americanus* and *Ancylostoma duodenale*, have been associated to impaired growth, deficit cognitive development and reduced education attendance [3]. Generally, individuals with low-to-moderate infections are asymptomatic, whereas heavy infections are associated with morbidity [3]. The World Health Organization (WHO) recommends deworming, or preventive chemotherapy, with albendazole (ALB) or mebendazole of pre-school age children (PSAC) and school-age children (SAC) in STH endemic areas (prevalence ≥ 20.0%) to achieve a coverage of 75% until 2020 [3].

Angola faced a 40-year period of war until 2002, with a massive destruction of infrastructures, forced migration, increased malnutrition, diseases and deaths [4]. Ten years later, in 2012, the government made a commitment to reducing stunting and wasting to less than 5% and underweight to less than 10% in children under-five years until 2021 [5]. However, according to the Multiple Indicator Health Survey 2015-2016, a large proportion of children are still suffering from stunting (37.6%) and wasting (4.9%) [6]. In Bengo province, Dande municipality, malnutrition was previously reported as one of

89 the major causes of deaths of under-five children between 2009 and 2012 [7]. In 2010, a
90 community-based study registered a prevalence of STH in PSAC of 22.3%, as well in
91 SAC (31.6%) and mothers (28.0%) [8]. Additionally, intestinal infections caused by
92 protozoa such as *Giardia lamblia* (21.6%) were also diagnosed in a hospital-based study
93 conducted in 2013 in Bengo [9]. To reduce morbidity from STH infections, the national
94 strategy includes preventive chemotherapy with ALB in SAC, along with access to better
95 hygiene and water conditions [10]. However, a single dose regimen of ALB has low
96 efficacy against *T. trichiura* (cure rate of 28%) and longer-regimens would be needed to
97 treat *Strongyloides stercoralis* and *G. lamblia,* previously associated with growth
98 impairment [11-14].

99 Although preventive chemotherapy has been shown to impact overall STH prevalence
100 levels in a variety of countries, the health benefits and cost-effectiveness of deworming
101 have been questioned by some. According to some systematic reviews, including the most
102 recent Cochrane Systematic Reviews published in 2015, there are no benefits of
103 deworming children living in endemic areas for STH at the community level (infected
104 and non-infected), including growth and haemoglobin levels [15, 16]. Similarly, no
105 significant effect of routine deworming was found on weight gain and on mortality of two
106 million PSAC in India [17]. Conversely, analysis of more than 320.000 PSAC from 45
107 countries found that those who were dewormed were less likely to be stunted and anaemic
108 [18]. Results are controversial and it is clear that further research with new approaches
109 are needed [19]. This longitudinal study was implemented to investigate if treatment of
110 intestinal parasites (with or without previous diagnosis) in two levels (individual or
111 household) impacts on nutritional status of children 2-5 years old, after a two-year follow-
112 up. Conducting at individual *versus* household level is important to understand if
113 including all household members can benefit children growth by preventing transmission

among family. Moreover, we considered the inclusion of children older than 24 months of age, not only because this is the age at which children become more frequently infected with intestinal parasites, but also to investigate the contribution of these different strategies in reducing chronic malnutrition beyond the well-known "window of opportunity" [20].

Material and Methods

Study design, setting and participants

This four-arm randomised parallel trial was conducted in the Dande Health and Demographic Surveillance System (HDSS) study area, implemented by the Health Research Centre of Angola (CISA), which includes Caxito, Mabubas, Úcua, and a small part of Kikabo communes, located in Dande municipality, Bengo province, Angola [21]. Enrolment was conducted between December 2013 and December 2014 at three outpatient health units in Caxito: Hospital Geral do Bengo, Hospital Municipal do Dande, and Posto Médico o Bom Samaritano.

At baseline, a questionnaire was applied in Portuguese to collect sociodemographic information, clinical condition and medication history. Anthropometric measurements of weight, height and mid-upper arm circumference (MUAC) were assessed. A single blood sample was collected for anaemia and malaria diagnosis, and a stool sample per child was requested for diagnosis of intestinal parasites.

In a first phase, children were deemed eligible according to inclusion criteria: age between 20-36 months at the recruitment (so during the follow-up period the age varied between 24 and 59 months); residence in the HDSS area; and no history of antibiotic or antiparasitic drug (previous ten days). Then, if an infection with at least one pathogenic

intestinal parasite was found, the child met all criteria to proceed in the study and was randomly allocated to one of four arms. Household members were also included in two of the four arms. At baseline, medication was provided according to the pathogenic agent identified before randomisation. Exclusion criteria was applied for children who did not appear to receive medication or whose parents did not consent their participation in the study. For household members, exclusion criteria included any person who did not live in the same house of the child, and those who refused to participate in the study.

Ethics statement

Medication for anaemia and infections caused by intestinal parasites and malaria was freely provided by clinical staff. Parents or caregivers were asked to sign two written consents (fingerprinted if illiterate) allowing the child to participate in the study: the first during recruitment and the second one for the follow-up period. Local authorizations and ethical approvals were obtained from the Bengo Provincial Health Authority, from the Ethics Committee of the Angolan Ministry, and from the IHMT Ethics Council, NOVA University of Lisbon, Portugal. Although the study protocol was submitted for ethical approvals before enrolment of the first participant, registration of the trial was performed retrospectively due to the lack of awareness of the mandatory prospective registration. Probably it would be easier and helpful if registration was also a requirement included in the national ethics committee. The trial was registered in ISRCTN registry, number ISRCTN72928001.

Randomisation and masking

Children were randomly assigned (1:1:1:1) by a nurse to one of the four arms: Arm 1 (A1), to receive a single annual dose of ALB 400 mg at individual level; Arm 2 (A2), to receive a single annual dose of ALB 400 mg at household level; Arm 3 (A3), to test-and-

161 treat pathogenic intestinal parasites every four months at individual level; and Arm 4
162 (A4), to test-and-treat pathogenic intestinal parasites every four months at household
163 level. Participants and clinical trial staff were aware of the group to which children were
164 assigned.

165 Common procedures between arms

166 Community follow-up (Fu) was performed at 4th (Fu1), 8th (Fu2), 12th (Fu3), 16th (Fu4),
167 20th (Fu5) and 24th months (Fu6) after inclusion of each child to collect information on
168 reported symptoms, anthropometric measurements, anaemia and malaria diagnosis.

169 Symptoms

170 Reported symptoms were related to the previous week and included fever, vomiting,
171 blood in the stool and diarrhoea (defined as three or more loose or liquid stools per day);

172 Anthropometric measurements

173 Weight (using scale seca® 877), height (using seca®213, with precision of 0.1 cm), and
174 MUAC measurements were assessed by trained health professionals. MUAC values were
175 transformed to standardized Z-score (MUACZ) using WHO ANTHRO Software (version
176 3.2.2) [22]. Additionally, weight and height measurements were converted to
177 anthropometric indices in Z-scores, according to WHO Child Growth Standards: height-
178 for-age (HAZ), weight-for-height (WHZ) and weight-for-age (WAZ) to identify stunting,
179 wasting and underweight, respectively. Children with Z-score≥-1 were considered
180 eutrophic. Malnutrition was classified as mild (-2≤Z-score<-1), moderate (-3≤Z-score<-
181 2), and severe (Z-score<-3). Children with symptoms of bilateral pitting oedema were
182 classified as acute severely malnourished [23]

183

Anaemia and malaria

A blood sample collected by finger prick was used to determine the haemoglobin (Hb) concentration using the HemoCue® Hb 301 System (HemoCue® AB, Angelhome, Sweden) and to classify anaemia as: no anaemia (≥11.0 g/dl), mild: (10.0-10.9 g/d), moderate (7.0-9.9 g/dl), and severe anaemia (<7.0 g/dl). Malaria diagnosis was performed by rapid immunochromatographic test (Standard Diagnostics Bioline Malaria Ag *P.f*/*P.v*, Standard Diagnostics Inc., Republic of Korea).

Interventions

Annual single dose of ALB

Annual single dose of ALB (Fu1 and Fu4) was performed in A1 and A2, at individual and household levels, respectively, and without any previous knowledge of infection status. Participants older than 24 months received a single dose of ALB 400 mg, while those between 12 months and 24 months received a single dose of ALB 200 mg.

At the end of the study, an additional stool sample was requested in participants of each arm (A1 and A2, at individual and household levels, respectively) for the diagnosis and treatment of intestinal parasite. We considered that it was ethically more appropriate and it would allow us to understand the pattern of infection among participants who received ALB during the follow-up. This did not influence the comparison of primary outcomes between the four arms since they were assessed before requiring the stool sample.

Test-and-treat intestinal parasites approach

Test-and-treat intestinal parasites approach (from Fu1 to Fu6) was performed in A3 and A4, at individual and household levels, respectively.

A stool sample per child in A3 or per household member in A4 was requested for microscopic detection of intestinal parasites using direct saline and iodine mounts, formalin-ether sedimentation technique (Parasite Recovery System, PRS^{TM}, Alphatec), modified Ziehl-Neelsen technique (for the identification of *Cryptosporidium* spp., *Isospora belli* and *Cyclospora cayetanensis* oocysts), and Kato-Katz smears (for the quantitative diagnosis of intestinal schistosomiasis and STHs within the 60 minutes of slide preparation Vertergaard Frandsen, Switzerland) [24]. If *Entamoeba histolytica/dispar* was identified through microscopy, a rapid test for the qualitative detection of *Entamoeba histolytica* was also performed (TECHLAB® E. HISTOLYTICA QUICK CHECK^{TM}, code T30409). Microscopic analysis was carried out by two blinded microscopists, and by a third one for discordant results. Participants with positive results received medication by clinical staff, in Hospital Geral do Bengo, and according to a clinical protocol (Table 1).

Table 1. Treatment of pathogenic intestinal parasites identified at the recruitment and during the follow-up in A3 and A4.

Type of infection *Intestinal parasites identified*	**Medication regimens**										
	12 <age < 24 months						**Age ≥ 24 months**				
	ALB	**MTZ**	**TNZ**	**PZQ**	**Dosage**	**Frequency**	**ALB**	**TNZ**	**PZQ**	**Dosage**	**Frequency**
Single infections											
Ascaris lumbricoides	x				200 mg	single dose	x			400 mg	single dose
Ancylostoma sp.	x				200 mg	single dose	x			400 mg	single dose
Entamoeba histolytica		x			35-50 mg/kg/d[c]	8/8h, 7d		x		50mg/kg[b]	3d
Giardia lamblia		x			15mg/kg/d	8/8h, 5d		x		50mg/kg[b]	single dose
Hymenolepis nana				x	25mg/kg	single dose			x	25mg/kg	single dose
Strongyloides stercoralis	x				200 mg	12/12h, 7d	x			400 mg	12/12h, 7d
Trichuris trichiura	x				200 mg	3d	x			400 mg	3d
Multiple infections											
G.lamblia +E.histolytica		x			35-50 mg/kg/d[c]	8/8h, 7d		x		50mg/kg[b]	3d
G.lamblia +A.lumbricoides	x				200 mg	5d	x			400 mg	5d
G.lamblia +T.trichiura	x				200 mg	5d	x			400 mg	5d
G.lamblia +A.lumbricoides +T.trichiura	x				200 mg	5d	x			400 mg	5d
G.lamblia +S.stercoralis	x				200 mg	12/12h, 7d	x			400 mg	12/12h, 7d
G.lamblia +H.nana				x	25mg/kg[a]	single dose			x	25mg/kg	single dose
		x			15mg/kg/d	8/8h, 5d		x		50mg/kg[b]	single dose
A. lumbricoides +S. stercoralis	x				200 mg	12/12, 7d	x			400 mg	12/12, 7d
T.trichiura +H.nana				x	25mg/kg[a]	single dose			x	25mg/kg	single dose
	x				200 mg	3d	x			200 mg	3d

ALB: Albendazole; MTZ: Metronidazole; TNZ: Tinidazole; PZQ: Praziquantel; d:day;

[a] Praziquantel is administered alone in the first day

[b] maximum dose is 2g.

[c] maximum dose is 750mg.

An additional stool sample was requested ten days after completing the recommended treatment to ensure its effectiveness. Children younger than 12 months, pregnant women, and women breastfeeding did not receive any of the four interventions [25].

Outcomes

The primary outcomes were height, weight, HAZ, WHZ, WAZ, and MUACZ, from baseline to 4, 8, 12, 16, 20, and 24 months of follow-up to assess growth. Secondary outcomes included the occurrence of infection by intestinal protozoa and helminths in all follow-up assessments in A3 and A4.

Statistical analysis

Sample size calculation for this longitudinal study was explored in GLIMMPSE software [26], based on a trend of HAZ across time and arms. A total of 152 participants with intestinal parasitic infections (38 per arm) were required to achieve a power of 80%, a type error 1 of 0.05, and applying the Hotelling-Lawley Trace test considering the posterior use of the parametric repeated measures in statistical analysis (S1 Appendix).

Intention-to-treat (ITT) analysis is widely recommended to avoid bias associated with non-random loss, preserving the benefit of randomisation [27]. Thus, all randomised participants were included, after a missing values treatment [28]. Analyses to primary outcomes started describing the proportion of subjects with missing values by arms and choosing different methods to handle missing data. A special attention was given to height due to facility in terms of interpretation. In cases where the missing value was flanked by valid observations, interpolation was used to height, since there is a monotonic increasing in their values. For the remaining values, we performed multiple imputation using the

248 Expectation Maximization, identifying the most plausible mechanism underlying our
249 data. Missing Completely at Random (MCAR) was tested using Little´s test [29]. After
250 an exploratory analysis and hypothesis tests, multivariate analysis was explored to
251 determine nutritional changes induced by interventions throughout the follow-up
252 assessments. Given the failure of the assumptions of the classic repeated measures, non-
253 parametric approaches were used for quantitative variables [30].

254 Student *t* test was applied to compare the means of two independent groups, and ANOVA
255 to compare the means of more than two groups. When requirements for the independent
256 samples were not met (homogeneity of variances checked by Levene test and normality
257 by Kolmogorov-Smirnov and Shapiro-Wilk tests), nonparametric Mann-Whitney-
258 Wilcoxon and Kruskall-Wallis were used instead. Paired t-test or Wilcoxon or Friedman
259 tests were used to compare two or more moments. McNemar and Q-Cochran tests were
260 applied for paired binary variables in two or more time points.

261 Initially, nonparametric rank-based methods were explored in the *nparLD* (R program)
262 [31] to address the key questions for each primary outcome: i) do the arms/treatments
263 have the same effect?; ii) Is the time profile flat or there is a trend over the follow-up
264 period?; iii) Are the effects of the treatments similar over time? This first rank-based
265 approach is robust to outliers and present a competitive performance for small sample
266 sizes [31]. However, other strategies are advantageous [28, 32]. Thus, linear mixed effect
267 models (LMM) and generalized estimating equations (GEE) for longitudinal data were
268 explored to reinforce our findings, using *lme*, *nlme* and *geepack* packages [28, 32].
269 Different correlation structures were considered in several LMM and GEE models. Plots
270 were explored using *gglopt2* package. Initial data analysis was done using IBM SPSS
271 Software, version 24 (IBM Corp, Armonk, NY, USA), and advanced modelling using R
272 Program. CONSORT 2010 guidelines were followed in this work (S1 Table).

Results

Between December 2013 and December 2014, 692 children were assessed for eligibility, of which 121 were included and randomly assigned to one of the four arms (Fig 1).

Fig 1. Consort flow chart.

Baseline characteristics

The mean age of overall children was 26.6 ± 4.86 months and 50.4% of them were female. Overall, the proportion of mothers without education level 11/116 (9.5%) was higher compared with fathers 2/115 (1.7%), p=0.04. The distribution of children, considering their mothers and fathers' age and education level, their household characteristics, and their access to water and sanitation conditions, was well balanced across arms. River was among the major water source used for drinking or bathing, and 19.7% of overall children did not have access to sanitation facilities at the household (Table 2).

Table 2. Baseline sociodemographic characteristics by study arms.

	Variable (n)	Categories[a]	A1 (n=29)	A2 (n=31)	A3 (n=31)	A4 (n=30)
Child	**Sex ratio** (n=121)	Female : male	0.81 : 1.00	0.72 : 1.00	1.38 : 1.00	1.31 : 1.00
	Age (n=121)	Mean ± SD, (months)	25.1 ± 4.57	27.7 ± 4.93	25.8 ± 4.90	27.6 ± 4.70
	Exclusively breastfed (n=114)	Mean ± SD, (months)	4.6 ± 1.80	5.4 ± 1.82	4.3 ± 2.1	4.7 ± 2.3
	Complementary feeding (n=111)	Mean ± SD, (months)	19.7 ± 3.9	19.6 ± 5.0	20.1 ± 4.1	21.0 ± 3.8
Mother	**Age** (n=114)	Mean ± SD, (years)	27.32 ± 6.44	28.97 ± 6.68	26.67 ± 5.05	30.42 ± 7.90
	Maternal education (n=116)	No education	3 (10.3)	2 (6.9)	0 (0.0)	6 (20.7)
		Primary	8 (27.6)	14 (48.3)	18 (62.1)	11 (37.9)
		Secondary or higher	18 (62.1)	13 (44.8)	11 (37.9)	12 (41.4)
	Studying and working status (n=116)	Do not study or work	5 (17.2)	7 (24.1)	8 (27.6)	5 (17.2)
		Only working	11 (37.9)	8 (27.6)	7 (24.1)	11 (37.9)
		Study and work	4 (13.8)	5 (17.2)	5 (17.2)	5 (17.2)
		Only studying	9 (31.0)	9 (31.0)	9 (31.0)	8 (27.6)
Father	**Age** (n=92)	Mean ± SD, (years)	31.44 ± 7.93	35.07 ± 10.49	30.67 ± 4.90	36.84 ± 10.12
	Paternal education (n=115)	No education	1 (3.4)	1 (3.6)	0 (0.0)	0 (0.0)
		Primary	3 (10.3)	5 (17.9)	6 (20.7)	6 (20.7)
		Secondary or higher	25 (86.2)	22 (78.6)	23 (79.3)	23 (79.3)
	Studying and working status (n=115)	Do not study or work	0 (0.0)	0 (0.0)	1 (3.4)	1 (3.4)
		Only working	21 (72.4)	22 (78.6)	19 (65.5)	23 (793)
		Study and work	6 (20.7)	6 (21.4)	7 (24.1)	4 (13.8)
		Only studying	2 (6.9)	0 (0.0)	2 (6.9)	1 (3.4)
Household	**Members per household** (N=117)	Mean ± SD	6.29 ± 2.29	6.20 ± 2.30	5.45 ± 1.59	5.97 ± 2.06
	Place of residence (n=121)	Urban	27 (93.1)	31 (100.0)	30 (96.8)	27 (90.0)
	Rooms (n=117)	≤ 3	23 (79.3)	17 (58.6)	25 (83.3)	22 (75.9)
		>3	6 (20.7)	12 (41.4)	5 (16.7)	7 (24.1)
	Wall (n=117)	Adobe	18 (62.1)	20 (69.0)	23 (76.7)	26 (89.7)
		Bricks	11 (37.9)	9 (31.0)	7 (23.3)	3 (10.3)
	Floor (n=117)	Earth/sand	5 (17.2)	4 (13.8)	3 (10.0)	3 (10.3)
		Cement/ceramic	24 (82.8)	25 (86.2)	27 (90.0)	26 (89.7)

	Mobile phone (n=117)	Yes	27 (93.1)	28 (96.6)	30 (100.0)	25 (86.2)
	Television (n=117)	Yes	25 (86.2)	26 (89.7)	26 (86.7)	26 (89.7)
	Public electricity (n=117)	Yes	21 (72.4)	27 (93.1)	25 (83.3)	21 (72.4)
	Freezer (n=117)	Yes	22 (75.9)	22 (75.9)	23 (76.7)	21 (72.4)
	Cable TV (n=117)	Yes	19 (65.5)	23 (79.3)	20 (66.7)	20 (69.0)
	Wheelbarrow(n=117)	Yes	14 (48.3)	18 (62.1)	16 (53.3)	15 (51.7)
	Radio (n=117)	Yes	16 (55.2)	12 (41.4)	14 (46.7)	13 (44.8)
	Motorcycle(n=117)	Yes	9 (31.0)	9 (31.0)	15 (50.0)	9 (31.0)
	Generator (n=117)	Yes	4 (13.8)	6 (20.7)	8 (26.7)	10 (34.5)
	Animals(n=117)	Yes	6 (20.7)	3 (10.3)	7 (24.1)	7 (24.1)
	Refrigerator(n=117)	Yes	5 (17.2)	4 (13.8)	6 (20.0)	6 (20.7)
	Car (n=117)	Yes	6 (20.7)	4 (13.8)	3 (10.0)	7 (24.1)
	Bicycle (n=117)	Yes	1 (3.4)	2 (6.9)	4 (13.3)	0 (0.0)
Water source and sanitation	**Drinking water source**[b] (n=117)	Improved	21 (72.4)	22 (75.9)	19 (63.3)	21 (72.4)
		Unimproved	8 (27.6)	7 (24.1)	11 (36.7)	8 (27.6)
	Drinking water source (n=117)	River	8 (27.6)	5 (17.2)	9 (30.0)	7 (24.1)
		Tap in the yard	5 (17.2)	12 (41.4)	7 (23.3)	8 (27.6)
		Private tank	9 (31.0)	6 (20.7)	9 (30.0)	10 (34.5)
		Others[c]	7 (24.1)	6 (20.6)	5 (16.7)	4 (13.8)
	Bath water source (n=117)	Irrigation channel	9 (31.0)	4 (13.8)	9 (30.0)	6 (20.7)
		River	7 (24.1)	6 (20.7)	9 (30.0)	8 (27.6)
		Tap in the yard	3 (10.3)	7 (24.1)	4 (13.3)	7 (24.1)
		Private tank	3 (10.3)	5 (17.2)	2 (6.7)	4 (13.8)
		Others[d]	7 (24.1)	7 (24.1)	6 (20.0)	4 (13.8)
	Latrine (n=117)	No facility	7 (24.1)	3 (10.3)	7 (23.3)	6 (20.7)
		Public	10 (34.5)	10 (34.5)	9 (30.0)	7 (24.1)
		Private	12 (41.4)	16 (55.2)	14 (46.7)	16 (55.2)

[a]For categorical variables characteristics are expressed as n (%);
[b] According to the WHO/UNICEF Joint Monitoring Programme for Water Supply, Sanitation and Hygiene (JMP) [32]. Improved water includes piped water into dwelling, tap in the yard, public tap water, tubewell, borehole, covered or uncovered tank, tanker truck; and unimproved water includes river, irrigation channel.
[c] includes piped water, irrigation channel, borehole and tubewell.
[d] includes piped water, borehole and tubewell.

At baseline, overall children had mean values of height (84.63cm±5.0) weight (11.26kg±1.79), HAZ (-1.34±1.33); WHZ (-0.29±1.18); WAZ (-0.80±0.98) and MUACZ (-0.88±0.98). On average, anthropometric indices of children were negative in all arms, with exception of WHZ in A3, as shown in Table 3.

Table 3. Baseline nutritional status, infection with intestinal parasites, symptoms reported, malaria, and anaemia in children by study arms.

	Variable (n)	**Categories**[a]	**A1** (n=29)	**A2** (n=31)	**A3** (n=31)	**A4** (n=30)
Nutritional status	**Height** (n=121)	Mean ± SD	83.63 ± 5.32	85.49 ± 5.89	84.19 ± 5.68	85.15 ± 6.73
	Weightb (n=119)	Mean ± SD	10.70 ± 1.74	11.58 ± 1.77	11.40 ± 1.70	11.48 ± 1.87
	MUAC (n=121)	Mean ± SD	13.83 ± 1.32	14.34 ± 1.12	14.33 ± 1.01	14.40 ± 0.94
	HAZ (n=121)	Mean ± SD	-1.26 ± 1.41	-1.35 ± 1.35	-1.30 ± 1.33	-1.43 ± 1.29
	WHZ[b] (n=119)	Mean ± SD	-0.65 ± 1.22	-0.25 ± 1.02	0.00 ± 1.36	-0.22 ± 0.98
	WAZ[b] (n=119)	Mean ± SD	-1.15 ± 1.19	-0.87 ± 1.07	-0.65 ± 1.15	-0.89 ± 1.04
	MUACZ (n=121)	Mean ± SD	-1.19 ± 1.21	-0.85 ± 0.93	-0.73 ± 0.90	-0.75 ± 0.84
	Stunting (n=121)	Eutrophic	12 (41.4)	11 (35.5)	11 (35.5)	10 (33.3)

		Mild	8 (27.6)	10 (32.3)	12 (38.7)	10 (33.3)
		Moderate	5 (17.2)	6 (19.4)	6 (19.4)	8 (26.7)
		Severe	4 (13.8)	4 (12.9)	2 (6.5)	2 (6.7)
	Wasting (n=121)	Eutrophic	17 (58.6)	23 (74.2)	24 (77.4)	22 (73.39
		Mild	8 (27.6)	6 (19.4)	6 (19.4)	6 (20.0)
		Moderate	3 (10.3)	1 (3.2)	1 (3.2)	1 (3.3)
		Severe	1 (3.4)	1 (3.2)	0 (0.0)	1 (3.3)
	Underweight (n=119)	Eutrophic	16 (55.2)	15 (50.0)	18 (58.1)	16 (55.2)
		Mild	7 (24.1)	12 (40.0)	7 (22.6)	9 (31.0)
		Moderate	4 (13.8)	2 (6.7)	6 (19.4)	3 (10.3)
		Severe	2 (6.9)	1 (3.3)	0 (0.0)	1 (3.4)
Pathogenic intestinal parasites	**Type of infection** (n=121)	Monoparasitism	25 (86.2)	26 (83.9)	28 (90.3)	26 (86.7)
		Polyparasitism	4 (13.8)	5 (16.1)	3 (9.7)	4 (13.3)
	Group of parasites (n=121)	Protozoa (P)	18 (62.1)	17 (54.8)	19 (61.3)	13 (43.3)
		Helminths (H)	8 (27.6)	12 (38.7)	11 (35.5)	14 (46.7)
		P + H	3 (10.3)	2 (6.5)	1 (3.2)	3 (10.0)
	Giardia lamblia (n=121)	Positive	17 (58.6)	18 (58.1)	19 (61.3)	15 (50.0)
	Ascaris lumbricoides (n=121)	Positive	3 (10.3)	8 (25.8)	8 (25.8)	12 (40.0)
	Strongyloides stercoralis (n=121)	Positive	5 (17.2)	3 (9.7)	4 (12.9)	4 (13.3)
	Trichuris trichiura (n=121)	Positive	2 (6.9)	2 (6.5)	1 (3.2)	2 (6.7)
	Hymenolepis nana (n=121)	Positive	1 (3.4)	3 (9.7)	3 (9.7)	0 (0.0)
	***Cryptosporidium* spp.** (n=121)	Positive	3 (10.3)	1 (3.2)	1 (3.2)	2 (6.7)
	Entamoeba histolytica (n=121)	Positive	2 (6.9)	1 (3.2)	0 (0.0)	0 (0.0)
Malaria, Hb,	**Malaria** (*P.falciparum*) (n=121)	Positive	4 (13.8)	3 (9.7)	2 (6.5)	2 (6.7)
	Haemoglobin (g/dl) (n=116)	mean ± SD	10.3 ± 2.06	10.5 ± 1.65	11.0 ± 1.05	10.5 ± 1.81
	Anaemia (n=119)	No	11 (40.7)	15 (48.4)	18 (58.1)	11 (36.7)
		Mild	7 (25.9)	7 (22.6)	9 (29.0)	13 (43.3)
		Moderate	7 (25.9)	7 (22.6)	4 (12.9)	4 (13.3)
		Severe	2 (7.4)	2 (6.5)	0 (0.0)	2 (6.7)
	Diarrhoea (n=121)	Yes	15 (51.7)	10 (32.3)	16 (51.6)	17 (56.7)
	Fever (n=121)	Yes	24 (82.8)	25 (80.6)	22 (71.0)	26 (86.7)
	Vomiting (n=121)	Yes	4 (13.8)	7 (22.6)	5 (16.1)	2 (6.7)

[a] For categorical variables characteristics are expressed as n (%).
[b] Did not include 2 children with bilateral oedema, one from A2 and another one from A4.

Moderate-to-severe malnutrition was 31.0% in A1, 32.3% in A2, 25.9% in A3 and 33.4% in A4. However, a great proportion was also classified as having mild malnutrition (27.6% in A1, 32.3% in A2, 38.7% in A3 and 33.3% in A4). Monoparasitism was more common in all arms compared with multiple infections. Intestinal protozoa (single or multiple) were the most frequent among arms, with exception of A4 where helminthic infections were more frequent. Considering the pathogenic agent, *Giardia lamblia* was the most frequent in the four arms. Of the 31 *Ascaris lumbricoides* positive samples, Kato-Katz was performed in 17 samples: 11 (64.7%) with light intensity and six (35.3%) with moderate-to-severe (data not shown).

Overall, malaria was diagnosed in 11 children (9.1%) at baseline and more than 50% presented mild-to-severe anaemia. Almost half of the children had diarrhoea at baseline (Table 3).

Loss to follow-up and missing values

Of the total, 12 (9.9%) children were permanently lost to the follow-up and did not perform the following assessment due to death (3), house not located (3), dropped-out (3), and emigration (3/12) (Fig 1). Temporary withdrawal occurred in children from all arms, mainly due to the absence of participants from their homes on the day the evaluation was scheduled (S2 Appendix). S2 Table presents complete primary outcomes and missing values. A total of 96 (79.3%) children conclude the study with complete data, whereas 25 (20.7%) had at least one missing value during the two years of the follow-up. No differences were found among arms (p=0.534).

Effect of interventions on nutritional outcomes

The primary analysis was ITT and involved all patients randomly assigned. Firstly, using parametric and non-parametric tests, the primary outcomes in each follow-up were not significantly different among arms.

From a descriptive point of view (S3 Table), on average, children from A1 (younger) had lower stature and weight at entry and persisted, however, no significant differences were found comparing the four arms. Considering mean values of HAZ, WHZ and WAZ, no differences were detected among arms in any follow-up assessment. Mean values of HAZ remained negative and far from zero in all six moments, highlighted in S3 Table (ranging from -1.42±1.19 to -0.99±0.98). Negative values persisted for WAZ (-1.00±1.03 to -0.57±0.85), and MUACZ (-0.95±0.85 to -0.59±0.82), whereas positive mean values were registered considering WHZ (ranging from -0.51±1.06 to 0.14±0.97) (S3 Table).

During the follow-up period, moderate-to-severe stunting in children varied between 19.4% and 36.7%, while mild-to-severe stunting ranged from 44.8% to 72.4% (values

presented in bold in S4 Table). Until the end of the study, a significant decrease was observed in mild-to-severe stunting (mainly in A1 by 27.6%, A2 by 19.3% and A4 by 20.0%), but not in moderate-to-severe stunting (although in A2 the reduction from 35.5% to 19.4% was close to be significant). Analysing the progress of stunting separated by sex, no differences were observed among arms and between the first and the sixth follow-ups (S5 Table). Moreover, no differences between the initial and final prevalence of wasting and underweight were registered within arms (S4 Table).

Regarding the three key questions (i, ii, and iii), analysing simultaneously the effects of *arm* (treatment), *time* (follow-up) and interaction *arm*time* on anthropometric outcomes, results of *npar*LD, LMM and GEE models are shown in Table 4.

Table 4. ANOVA tables for primary outcomes, using a nonparametric analysis of longitudinal data (nparLD) and LMM and GEE models.

Outcome	NparLD						LMM				GEE		
	ANOVA modified			Wald			ANOVA				ANOVA		
Effect	Statistic	df	*p*-value	Statistic	df	*p*-value	Statistic	df_1	df_2	*p*-value	Statistic[a]	df	*p*-value
Height													
Arm	0.94	2.930	0.42	3.03	3	0.39	1.0	3	117	0.48	10.0	3	0.02*
Time	1210.04	1.903	<0.001*	1860.84	5	<0.001*	6997.0	1	601	<0.001*	427.0	1	<0.001*
Arm*Time	0.79	5.483	0.57	11.86	15	0.69	0.0	3	601	0.72	0.0	3	1.00
Weight													
Arm	0.63	2.939	0.59	1.82	3	0.61	1.0	3	117	0.62	7.5	3	0.06
Time	474.71	3.279	<0.001*	1053.28	5	<0.001*	2025.0	1	601	<0.001*	216.8	1	<0.001*
Arm*Time	0.82	8.979	0.60	15.83	15	0.39	0.0	3	601	0.69	0.1	3	0.99
HAZ													
Arm	0.06	2.986	0.98	0.19	3	0.98	0.1	3	117	0.96	2.13	3	0.55
Time	29.30	3.256	<0.001*	64.53	5	<0.001*	54.2	1	601	<0.001*	5.23	1	0.02*
Arm*Time	0.73	8.670	0.68	11.43	15	0.72	0.4	3	601	0.78	0.13	3	0.99
WAZ													
Arm	0.12	2.986	0.95	0.40	3	0.94	0.3	3	117	0.84	5.08	3	0.17
Time	6.06	4.292	<0.001*	23.73	5	<0.001*	1.9	1	601	0.17	0.21	1	0.65
Arm*Time	0.77	10.112	0.66	15.92	15	0.39	0.3	3	601	0.83	0.04	3	1.00
WHZ													
Arm	0.29	2.955	0.83	0.95	3	0.81	0.5	3	117	0.68	8.10	3	0.04*
Time	10.15	4.534	<0.001*	46.32	5	<0.001*	15.6	1	601	<0.001*	4.08	1	0.04*
Arm*Time	0.48	11.766	0.93	9.63	15	0.84	0.06	3	601	0.98	0.04	3	1.00
MUACZ													
Arm	0.35	2.947	0.79	1.00	3	0.80	0.5	3	117	0.66	7.52	3	0.06
Time	4.35	4.549	0.001*	19.15	5	0.002*	6.8	1	601	0.009*	1.86	1	0.17
Arm*Time	0.40	12.047	0.96	6.80	15	0.96	1.0	3	601	0.39	0.77	3	0.86

[a]Statistic: $\chi 2$; HAZ: height-for-age Z-score; WAZ: weight-for-age Z-score; WHZ: Weight-for-height Z-score; MUACZ: Mid-Upper Arm Circumference Z-score; *$p<0.05$*

Considering *npar*LD, no significant effects were obtained in any of the interventions (effect of arm), nor by arm*time interaction. However, temporal changes (effect of time) occurred in all nutritional outcomes ($p<0.05$) (Table 4).

Exploring the same questions using models GEE and LMM models, the same patterns were observed (Table 4). Nevertheless, there was a significant effect of arm intervention on height (p=0.02) and WHZ (*p*=0.04), and very close to be significant on weight and MUACZ. Considering A1 as a reference, parameter estimations associated to GEE and LMM models are presented in Table 5.

Table 5. Parameter estimation for primary outcomes using GEE and LMM models

Variable	Parameter	LMM					GEE			
		E	SE	Df	Statistic	*p*-value	E	SE	Statistic	*p*-value
Height	(Intercept)	84.1	1.08	601	78.1	<0.001*	84.3	0.92	8324.2	<0.001*
	A1A2	2.1	1.50	117	1.4	0.16	2.1	1.32	2.6	0.11
	A1A3	0.3	1.50	117	0.2	0.85	0.3	1.26	0.1	0.80
	A1A4	1.5	1.51	117	1.0	0.33	1.4	1.44	1.0	0.32
	Time	2.5	0.06	601	40.9	<0.001*	2.5	0.24	106.67	<0.001*
	A1A2:Time	0.0	0.09	601	0.0	0.97	0.0	0.34	0.0	0.95
	A1A3:Time	0.1	0.09	601	0.7	0.48	0.1	0.33	0.0	0.85
	A1A4:Time	0.0	0.09	601	-0.4	0.69	0.0	0.38	0.0	0.98
Weight	(Intercept)	11.4	0.35	601	32.1	<0.001*	11.4	0.32	1301.5	<0.001*
	A1A2	0.6	0.49	117	1.1	0.26	0.6	0.44	1.8	0.18
	A1A3	0.2	0.49	117	0.5	0.62	0.3	0.41	0.4	0.51
	A1A4	0.4	0.50	117	0.8	0.45	0.4	0.46	0.8	0.36
	Time	0.6	0.03	601	21.3	<0.001*	0.6	0.09	45.1	<0.001*
	A1A2:Time	0.0	0.04	601	0.2	0.87	0.0	0.12	0.0	0.98
	A1A3:Time	0.0	0.04	601	0.9	0.38	0.0	0.11	0.1	0.81
	A1A4:Time	0.0	0.04	601	1.0	0.34	0.0	0.13	0.0	0.85
HAZ	(Intercept)	-1.38	0.210	601	-6.6	<0.001*	-1.39	0.201	47.7	<0.001*
	A1A2	0.04	0.292	117	0.1	0.90	0.04	0.278	0.0	0.90
	A1A3	0.00	0.292	117	0.0	0.99	0.01	0.271	0.0	0.98
	A1A4	-0.09	0.295	117	-0.3	0.77	-0.09	0.280	0.1	0.76
	Time	0.04	0.015	601	2.8	0.01*	0.04	0.051	0.6	0.42
	A1A2:Time	0.02	0.021	601	1.0	0.31	0.03	0.069	0.1	0.72
	A1A3:Time	0.02	0.021	601	0.7	0.46	0.02	0.068	0.1	0.83
	A1A4:Time	0.01	0.021	601	0.6	0.56	0.02	0.071	0.1	0.83
WHZ	(Intercept)	-0.08	0.179	601	-0.4	0.67	-0.08	0.181	0.2	0.65
	A1A2	0.06	0.248	117	0.2	0.81	0.08	0.250	0.1	0.75
	A1A3	0.25	0.248	117	1.0	0.32	0.26	0.226	1.3	0.25
	A1A4	0.11	0.250	117	0.4	0.67	0.13	0.240	0.3	0.58
	Time	-0.04	0.022	601	-2.0	0.04*	-0.04	0.046	0.7	0.41
	A1A2:Time	0.00	0.030	601	-0.1	0.91	-0.01	0.062	0.0	0.87
	A1A3:Time	0.00	0.030	601	0.1	0.94	-0.00	0.058	0.0	0.98
	A1A4:Time	0.01	0.030	601	0.3	0.78	0.00	0.062	0.0	1.00
WAZ	(Intercept)	-0.79	0.175	601	-4.5	<0.001*	-0.80	0.178	20.2	<0.001*
	A1A2	0.04	0.243	117	0.2	0.86	0.06	0.244	0.1	0.79
	A1A3	0.18	0.243	117	0.7	0.47	0.19	0.228	0.7	0.40
	A1A4	0.01	0.245	117	0.0	0.97	0.04	0.236	0.0	0.88
	Time	-0.02	0.016	601	-1.4	0.16	-0.02	0.046	0.1	0.73
	A1A2:Time	0.02	0.022	601	0.7	0.47	0.01	0.061	0.0	0.88
	A1A3:Time	0.01	0.022	601	0.5	0.65	0.01	0.058	0.0	0.93
	A1A4:Time	0.02	0.022	601	0.9	0.38	0.01	0.060	0.0	0.86
MUACZ	(Intercept)	-0.92	0.153	601	-6.0	<0.001*	-0.92	0.168	30.1	<0.001*
	A1A2	0.24	0.213	117	1.1	0.27	0.24	0.236	1.0	0.31
	A1A3	0.35	0.213	117	1.7	0.10	0.35	0.120	3.1	0.08
	A1A4	0.36	0.215	117	1.7	0.09	0.36	0.215	2.8	0.09
	Time	0.00	0.021	601	0.2	0.86	0.01	0.042	0.0	0.91
	A1A2:Time	-0.03	0.029	601	-1.1	0.27	-0.03	0.058	0.3	0.57
	A1A3:Time	-0.04	0.029	601	-1.5	0.13	-0.04	0.051	0.7	0.41
	A1A4:Time	-0.04	0.029	601	-1.5	0.13	-0.04	0.054	0.7	0.44

Reference class = A1; E=Estimate; SE=Standard error; *p<0.05

363 The results were very similar, even for different correlation structures, enhancing
364 significant temporal changes in height and weight, regardless the method performed.
365 According to GEE analysis, based on population-average, children from A2 are estimated
366 to have 2.1 cm (SE=1.32) more than children from A1, while those from A4 and A3 are
367 estimated to have 1.4 cm (SE=1.44) and 0.3 (SE=1.26) more than children from A1,
368 respectively, Table 5. These estimates were identical to parameter estimation obtained in
369 LMM model. In terms of height, an increase of 2.5 cm is expected per four-months. The
370 significance of parameter associated to time in HAZ and WHZ outcomes were different
371 in two type of models. Fig 2 shows the estimates of the Relative Treatment Effects (RTE)
372 of each arm over time obtained by the rank-based approach for some of the primary
373 outcomes (for overall and by sex).

374 **Fig 2. Estimates of the relative effects of arm over time for primary outcomes to**
375 **overall and by gender.**

376 An increase in the effect seems clear for height over time. An almost overlap of lines is
377 visible for HAZ over time, supporting the non-significant finding from GEE models.
378 Indeed, plots from *npar*LD approach also show a change in the trajectories of HAZ and
379 WHZ, at least in Fu5 and Fu6 and by gender (Fig 2).

380 In terms of WAZ and WHZ, from Fu4 to Fu6, a decrease was observed, particularly in
381 A2. By sex, also without significance differences, A2 presented worse results in females,
382 namely for WAZ and WHZ.

383

384

Effect of test-and-treat intestinal parasites approach on secondary outcomes

There were no significant differences of intestinal infection frequencies between A3 and A4 during follow-up (S1 Fig). From Fu1 to Fu6, there was a significant reduction of intestinal parasitic infection in A4 (p=0.039), but not in A3 (p=0.727). *Giardia lamblia* was the most frequent parasite identified in each follow-up (S1 Fig). Similarly, the reduction of infection from Fu1 to Fu6 was higher in A4 (from 37.5% to 16.7%, *p*=0.07) than A3 (from 34.6% to 30.4%, *p*=0.727) (Fig 3). Conversely, *A. lumbricoides* infection was higher in A4 than A3, except in Fu6, although without significant differences: Fu1 (3.8 vs 4.2%), Fu2 (4.5% vs 12.5%), Fu3 (4.0% vs 23.1%), Fu4 (4.2% vs 16.7%), Fu5 (13.6% vs 20.0%), and Fu6 (8.7 vs 8.3%).

Fig 3. Stool sample collection and treatment of pathogenic intestinal parasites in A3 and A4 during follow-up. Protozoa, helminths or protozoa + helminths (P+H) include single and multiple infections.

Giardia lamblia and *A.lumbricoides* infections also include single and multiple infections. At the end of the study, we verified lower infection frequencies of *G. lamblia* in A4 (16.7%), compared with A3 (30.4%), A1 (34.8%), and A2 (40%), whereas infections caused by *A. lumbricoides* remained similar in all arms (near 9% for A1 and A2, 10% in A3, and 8% in A4) (Fig 4).

Fig 4. Percentage (%) of monoparasitism and polyparasitism at Fu0 and Fu6, highlighting *Giardia lamblia* and *Ascaris lumbricoides* infections.

Discussion

We investigated the impact of four interventions (annual albendazole at individual level; annual albendazole at household level; four-monthly test-and-treat intestinal parasites at individual level; and four-monthly test-and-treat intestinal parasites at household level on nutritional outcomes of children.

Malnutrition is a public health problem in Bengo

At baseline, a very high proportion of stunting was found among overall children (30.6%), (ranging from 25.9% to 33.9% among arms), similar to the estimates for Africa (30.3%) [1], but lower than the national (37.6%) and Bengo (39.7%) prevalence levels previously reported [6]. This was probably because the majority of participants was from urban areas, known to have lower prevalence of stunting compared with rural areas [6]. The percentage of wasting in overall children was 7.4%, very close to the Africa region estimates (7.1%), but higher when compared to MICS prevalence levels (4.7% for Bengo and 4.9% for the country-level) [1, 6]. This was possibly because children were recruited in health units seeking for healthcare services.

At baseline, overall mean HAZ (-1.34±1.33) was similar to the pattern described in a previous research including children from 57 countries (-1.43±1.70) and also without sex differences [33]. Growth faltering has been reported in low- and middle-income countries where children are already born with mean values of HAZ below the WHO reference and continue to decrease substantially until two years of age, after which it increase slightly until five years [20]. Considering children between two and five years, sub-Saharan Africa (where Angola is also included) is the region with the shortest children, after South Asia, while the tallest are in Europe and Central Asia with almost 1.5 Z-score higher [20].

Interventions and primary outcomes

After two years, this longitudinal study suggested that screening and treating intestinal parasites did not result in better growth outcomes of children compared with yearly albendazole, with exception of height and WHZ using GEE model at 5% level. Significant temporal changes were obtained in arms for all primary outcomes, regardless of the statistical analysis performed (*npar*LD and LMM models). The effect of time was not significant in GEE models for WAZ and MUACZ.

LMM and GEE models estimate an expected four-monthly increase of 0.04 in HAZ (≈0.01 per month), slightly higher than the catch-up growth reported for African children older than 24 months (0.005 z-score per month) [20]. Furthermore, the aforementioned study [20] reported a decrease of WAZ between -0.01 and -0.02 per month after 12 months of age, while in this study a smaller decrease was estimated (≈-0.005 WAZ per month). From Fu1 to Fu3, boys had lower mean of WHZ than girls, but at Fu4 there was an inversion of sex distribution, which was previously described for children between 44 and 59 months [33].

Although there was no evidence of significant effect on nutritional outcomes by any of the treatment strategies, an important reduction of mild malnutrition occurred in children from A1, A2 and A4. This is important since all levels of malnutrition, including mild malnutrition, have been previously associated with significantly higher mortality [34]. Although Angola has been at peace since 2002, malnutrition remains a public health problem. Children with mild malnutrition living in precarious environmental conditions with reduced access to improved hygiene and sanitation conditions and healthcare services can be at risk of increasing the severity of malnutrition.

Intestinal parasites and secondary outcomes

In Bengo, *G. lamblia* infection was previously reported in PSAC with diarrhoea (21.6%) [9]. In this study, it was the most frequent parasite diagnosed at baseline (57.0%), with the greatest proportion among children without diarrhoea, similar to precedent findings [35, 36]. There is evidence that subclinical infection of *G. lamblia* is negatively associated with growth in low resource settings, highlighting the importance of diagnosing and treating *G. lamblia* to control the spread of infection, and, consequently, its impact on nutritional status [13]. In A3 and A4, where screening was performed four-monthly, *G. lamblia* was the most frequent parasite identified in each follow-up. However, a greater reduction of *G. lamblia* occurred in A4, enhancing the importance of transmission among individuals living in the same household, as previously reported in Malaysia [37].

A higher rate of infection with *A. lumbricoides* was found compared with a previous community-study performed in Bengo (25.6 vs 15.3%) [8]. However, STH infections were mostly of light intensity and evidence suggests that those who are lightly infected or not infected do not benefit from deworming [3]. This could explain the lower impact of treatment on growth on this trial. *S. stercoralis* was the second most frequent helminth at baseline, highlighting the importance of this neglected STH.

Limitations

First, implementing a longitudinal study in a poor setting is challenging, and not surprisingly our sample was slightly smaller than the sample size calculated. One of the reasons was the higher percentage of children not delivering the stool sample at the recruitment, as well as those children who met the inclusion criteria but did not appear. Logistically it was not possible to extend the recruitment for longer periods. Second, diagnosis of intestinal parasites was performed using a single stool sample in each follow-

up, which could have contributed to underestimate the infections. However, collecting three samples instead of one would be logistically more difficult for caregivers, and would increase the lost to follow-up. To overcome this limitation, different laboratory techniques were performed. Third, there is no guarantee that participants did not take any other medication. Some treatments in A3 and A4 included more than one dosage/days, and we cannot guarantee that caregivers have complied with the prescription. Self-reported data from questionnaires are also prone to social desirability response bias (a limitation difficult to overcome in any study). Fourth, adherence to interventions throughout the study was higher in ALB-arms compared with test-and-treat arms, which could have contributed to bias. To overcome this limitation, an intention-to-treat analysis was performed according to CONSORT guidelines – S1 Table [27]. The greatest difficulty was centred on the delivery of samples, especially when household family members were also included. Follow-up visits can interfere with the dynamics of each household member, their leisure time, work schedule and responsibilities to the community. Besides, the access to some houses was difficult given the poor condition of roads. Thus, it is extremely important to be aware of the social context of the study. Lastly, given its design, the study cannot be generalized to the entire population.

Strengths

This trial includes seven repeated measurements from the same participant (including baseline), which is a key strength of studies with this design. A study with repeated measurements allows to chart the profile of the same individual across time, an advantage compared with cross-sectional study where different subjects are commonly observed at different times [30]. It is true that missing values can represent a huge challenge in a longitudinal study. However, different methods were applied for imputation and given

503 the fact that data from the same children was collected repeatedly, it decreased the risk of
504 incorrect anthropometric data since height does not decrease and subjects can serve has
505 their own controls. Moreover, a study with repeated measurements increases statistical
506 power for detecting changes across time, and smaller sample sizes are needed compared
507 with cross-sectional studies [38]. The main findings of this study were obtained by three
508 different statistical approaches for longitudinal data, one of them (*npar*LD) described in
509 the literature as robust to outliers and suitable for small sample sizes [31].

510 Conclusions

511 In conclusion, no significant effects were found for different interventions on nutritional
512 outcomes of children. Previous studies addressing parasitic infections and malnutrition in
513 Angola, were mainly cross-sectional and without any type of longitudinal intervention.
514 This trial contributes with new research approaches by including both intestinal protozoa
515 and helminths when thinking on therapeutic interventions to improve the growth of
516 PSAC. Screening and treating parasitic infections is more difficult and expensive than
517 preventive chemotherapy, especially in these settings. From an epidemiological
518 perspective, it is crucial to know the causal agent of an infection, in order to plan
519 preventive measures, and to provide the access to antimicrobial treatment, especially
520 when children are repeatedly exposed to a wide variety of pathogens. Besides, additional
521 research is needed to address the benefits of these interventions on nutrition outcomes of
522 children, ideally in a more heavily infected setting and including a higher number of
523 participants. Malnutrition can result from multiple factors and, thus, its reduction seems
524 to require multidisciplinary approaches including maternal and child interventions, safe
525 water and sanitation, access to health care services, food production, availability and
526 distribution, some of them not addressed in this study.

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Supporting information captions

S1 Appendix. Sample size calculation

S2 Appendix. Losses to follow-up

S3 Appendix. Study protocol nº 13-2013-TD (English)

S4 Appendix. Study protocol nº 13-2013-TD (Portuguese)

S1 Table. Consort 2010 checklist

S2 Table. Primary outcomes missing data by study arm

S3 Table. Mean primary outcomes during follow-up by arms and overall

S4 Table. Percentage of stunting, wasting and underweight in children during follow-up and by arms

S5 Table. Percentage of stunting during follow-up and by gender.

S1 Fig. Infection with pathogenic intestinal parasites in children during follow-up: A3 and A4. The proportion of children infected slightly fluctuated throughout the study period in both arms. There was a reduction of infection between Fu1 and Fu2 in children from A3 (from 42% to 36%), and A4 (from 58% to 33%). After, at Fu3, an increase of positive results was

694 observed, achieving 56% (14/25) in A3 and 54% (14/26) in A4. From that moment on, positive
695 cases slightly declined until the last follow-up, reaching percentages of 39% and 33% in A3 and
696 A4, respectively.

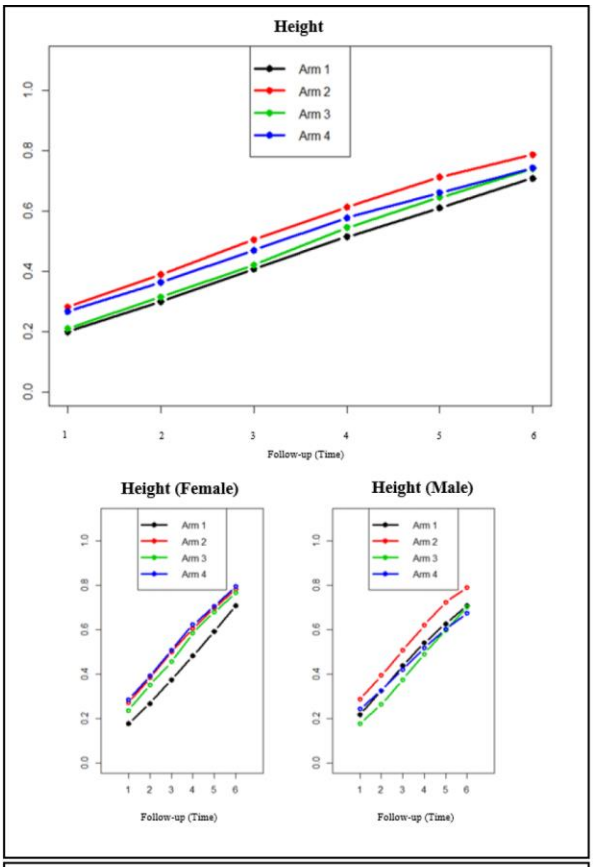
Fig 1. Consort flow chart.

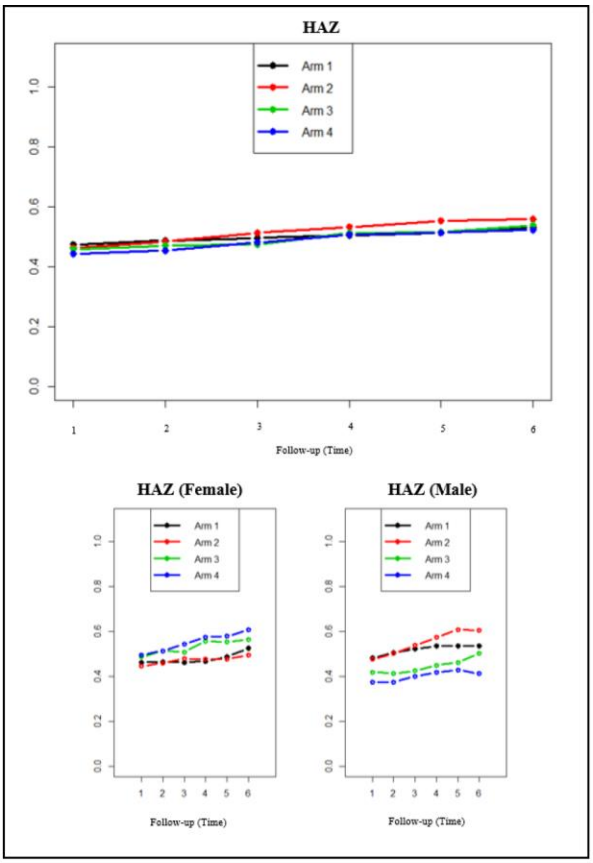
Common procedures include anthropometric measurements (weight, height, MUAC), and clinical questionnaire on symptoms, haemoglobin assessment and malaria diagnosis.

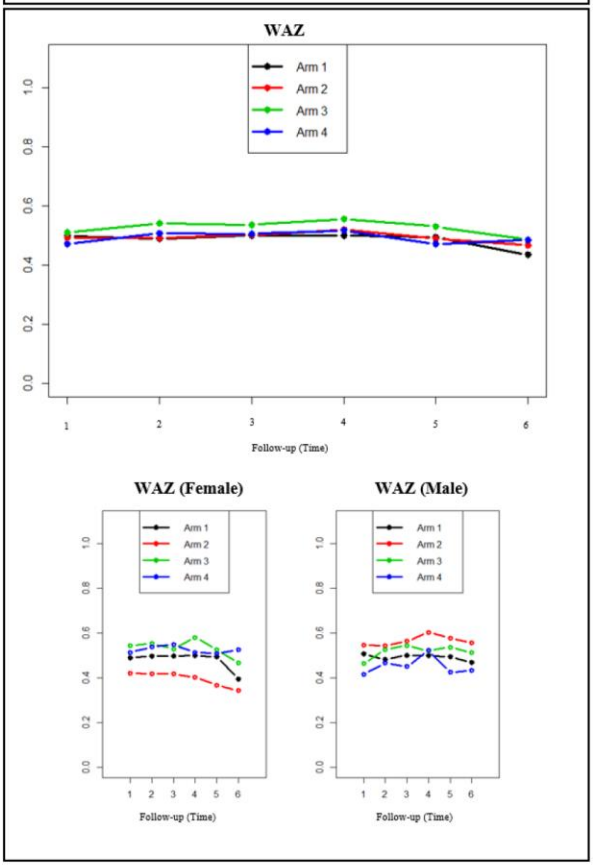
*Excluded from the following follow-up; mo: months after allocation; : Single dose of albendazole; : Stool sample collected; P+: Positive samples for pathogenic intestinal parasites; T: children infected with intestinal parasites who received treatment

: children included; : household members

Fig 2. Estimates of the relative effects of arm over time for primary outcomes to overall and by gender


Height
Arm 1
Arm 2
Arm 3
Arm 4
Follow-up (Time)
Height (Female)
Height (Male)


HAZ
Arm 1
Arm 2
Arm 3
Arm 4
Follow-up (Time)
HAZ (Female)
HAZ (Male)


WAZ
Arm 1
Arm 2
Arm 3
Arm 4
Follow-up (Time)
WAZ (Female)
WAZ (Male)

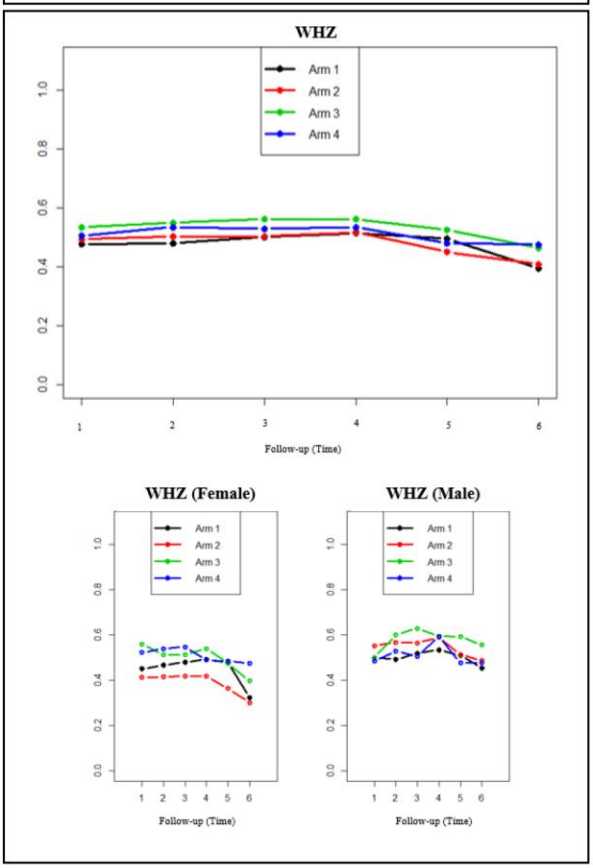

WHZ
Arm 1
Arm 2
Arm 3
Arm 4
Follow-up (Time)
WHZ (Female)
WHZ (Male)

Fig 3. Stool sample collection and treatment of pathogenic intestinal parasites in A3 and A4 during follow-up.

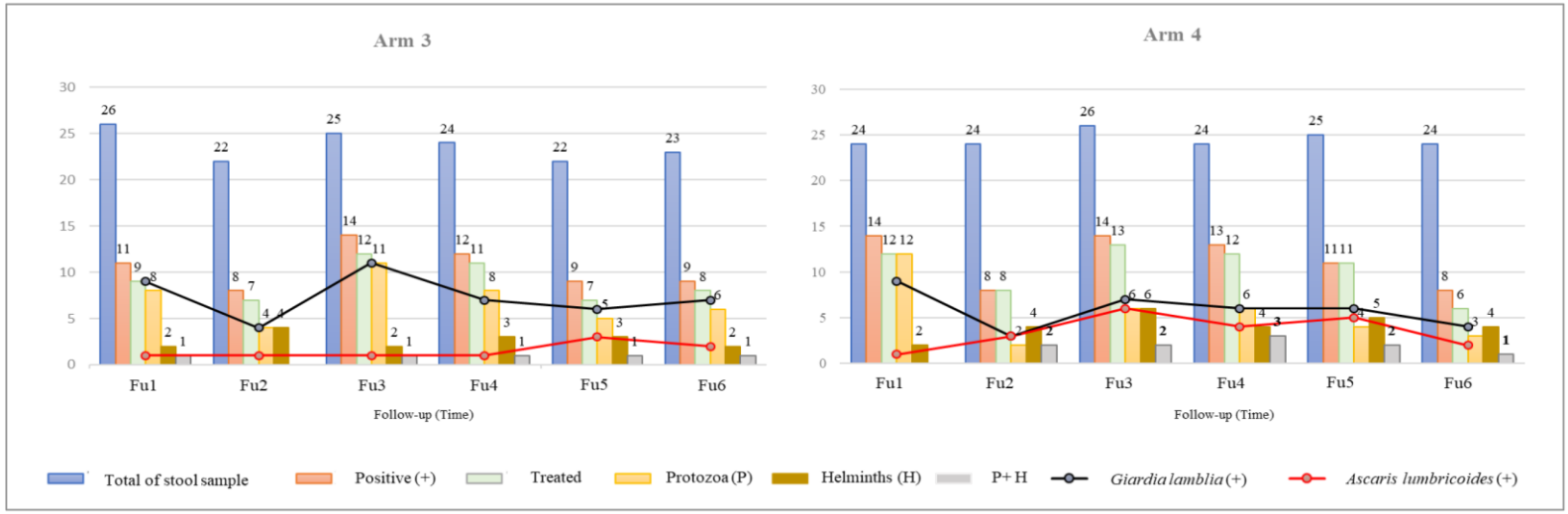
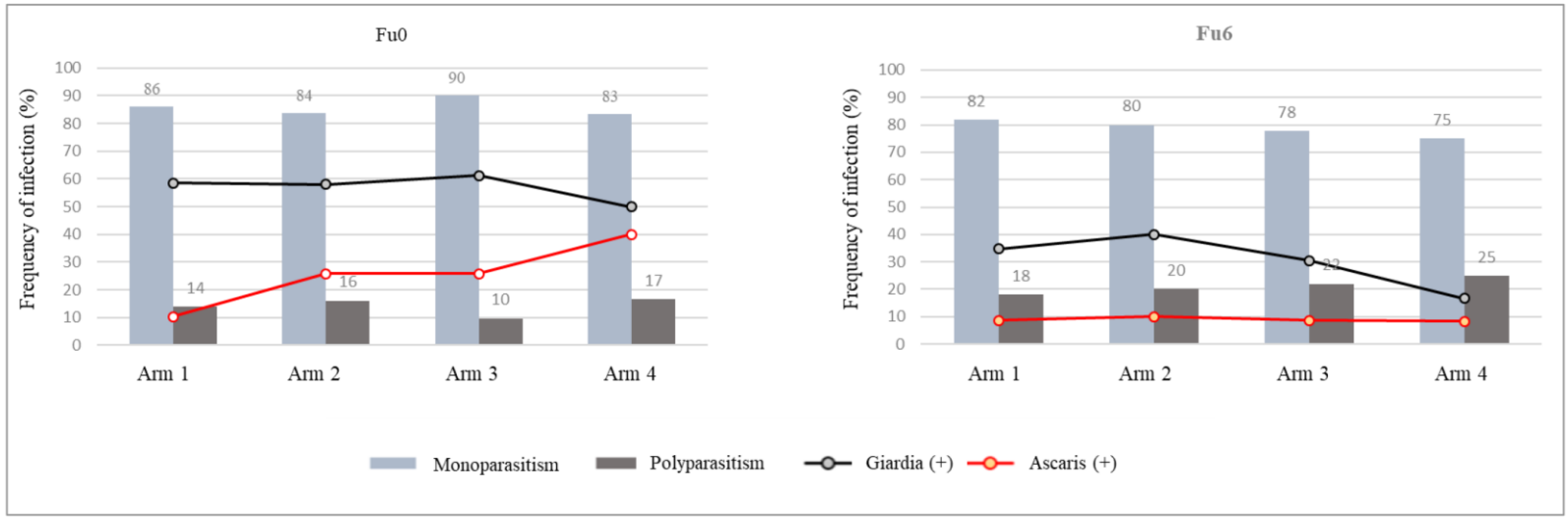

Arm 3
Arm 4
Fu1
Fu2
Fu3
Fu4
Fu5
Fu6
Follow-up (Time)
Total of stool sample
Positive (+)
Treated
Protozoa (P)
Helminths (H)
P+ H
Giardia lamblia (+)
Ascaris lumbricoides (+)

Fig 4. Percentage (%) of monoparasitism and polyparasitism at Fu0 and Fu6, highlighting *Giardia lamblia* and *Ascaris lumbricoides* infections.


Fu0
Frequency of infection (%)
100
90
80
70
60
50
40
30
20
10
0
86
84
90
83
14
16
10
17
Arm 1
Arm 2
Arm 3
Arm 4
Fu6
Frequency of infection (%)
82
80
78
75
18
20
22
25
Arm 1
Arm 2
Arm 3
Arm 4
Monoparasitism
Polyparasitism
Giardia (+)
Ascaris (+)

S1 Appendix. Sample size calculation

The sample size calculation was performed using GLIMMPSE software (URL: http://glimmpse.samplesizeshop.org/#/).This is an open source tool that researchers can use for computing the sample size or power of longitudinal studies with repeated measurements [1-3]. HAZ was considered the main response variable in our study, which is a continuous variable measured 7 times (in Z-scores): baseline, Fu1, Fu2, Fu3, Fu4, Fu5 and Fu6.

Step by step

The easy way to start is choosing the Guided Study Design Method, which is designed for applied researchers as physicians, nurses and other researchers. To calculate the sample size, the following criteria were defined and introduced in the software:

1. *Power value* – Usually a power value of 0.8 or 0.9 is desired. Considering the costs, human resources, logistic and time, in our study we chose *0.8* for calculation.

2. *Model* – On the repeated measurements we wrote *time* in dimension text box to indicate that we will take repeated measurements across time. HAZ is going to be assessed 7 times (including baseline and six community follow-ups) with equal spacing between them. The two main predictors defined were *treatment* and *level*. For *treatment* predictor we insert two categories: *Treatment without previous diagnosis* and *Treatment after diagnosis*. For *level* predictor, the categories are: *individual* and *household*. After that, a ratio 1:1:1:1 was chosen to obtain equal group sizes for arms.

3. *Hypothesis – selecting the primary hypothesis, a statistical test and a type 1 error rate*: In this study we aimed to evaluate the trend of the height-for-age Z score

reduction across time and within-participants-factors (treatment) using the Hotelling-Lawley Trace test, and a type error 1 of 0.05.

4. *Mean values* – In this step the researcher has to define which is the expected mean value for the response variable in each treatment group and at each follow-up. We assumed that the average at baseline was the same for all the study groups (A1, A2, A3 and A4), and for this reason we input 0 for the expected baseline reduction value. We expect that each month the HAZ will reduce 0.01, 0.02, 0.04 and 0.06 per month after *individual treatment without previous diagnosis* (A1), *household treatment without previous diagnosis* (A2), *individual treatment after diagnosis* (A3), *and household treatment after diagnosis* (A4), *respectively*.

5. *Standard deviations and correlations* – We considered a standard deviation (SD) of 1.1 Z-scores. For correlations between two consecutive measurements we assumed 0.95 with a decay rate of 1% for the remaining.

6. Results – The total sample size of children who are infected at least with one pathogenic intestinal parasite (protozoa and/or helminths) was computed to be 152 (38 participants per group).

References:

1. Kreidler SM, Muller KE, Grunwald GK, Ringham BM, Coker-Dukowitz ZT, Sakhadeo UR, et al. GLIMMPSE: Online Power Computation for Linear Models with and without a Baseline Covariate. Journal of statistical software. 2013;54(10). Epub 2014/01/10. PubMed PMID: 24403868; PubMed Central PMCID: PMCPMC3882200.
2. Guo Y, Logan HL, Glueck DH, Muller KE. Selecting a sample size for studies with repeated measures. BMC medical research methodology. 2013;13:100. Epub 2013/08/02. doi: 10.1186/1471-2288-13-100. PubMed PMID: 23902644; PubMed Central PMCID: PMCPMC3734029.
3. Guo Y, Pandis N. Sample-size calculation for repeated-measures and longitudinal studies. American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics. 2015;147(1):146-9. Epub 2014/12/24. doi: 10.1016/j.ajodo.2014.10.009. PubMed PMID: 25533082.

S2 Appendix. Losses to follow-up

Overall 12 (9.9%) were permanently lost to the follow-up (7 boys and 5 girls), and did not perform the following assessment, mainly due to death (3/12), house not located (3/12), dropped-out (3/12), and emigration (3/12). All deaths occurred in female children, with 32, 29 and 49 months old, and the cause of death was known for only one child belonging to Arm 3 (malaria). Temporary withdrawal occurred in children from all arms:

In Arm 1:

- ✓ 96.6% (28/29) of children received a single dose of ALB at Fu1;
- ✓ 85.7% (24/28) of children received a single dose of ALB at Fu4 (4 children were absent from their residence);
- ✓ Of the total included, 82.8% (24/29) received complete intervention in both Fu1 and Fu4.

In Arm 2:

- ✓ 90.3% (28/31) of children and 83.3% (125/150) of household members received a single dose of ALB at Fu1; Coverage of ALB by household was ≥ 80% in 25 cases.
- ✓ 93.1% (27/29) of children and 80.9% (123/152) of household members received a single dose of ALB at Fu4; Coverage of ALB by household was ≥ 80% in 23 cases.
- ✓ 71.0% (22/31) of children received ALB in both Fu1 and Fu4 with a household coverage ≥80%.

In Arm 3:

- ✓ The percentage of children delivering a stool sample and receiving appropriate treatment by follow-up was: 77.4% (24/31) in Fu1; 67.7% (21/31) in Fu2; 74.2% (23/31) in Fu3; 74.2% (23/31) in Fu4; 64.5% (20/31) in Fu5, and 71.0% (22/31) in Fu6.
- ✓ However, only 48.4% (15/31) of children delivered a stool sample and received appropriate treatment in all follow-ups.

In Arm 4:

- ✓ The percentage of children delivering a stool sample and receiving appropriate treatment by follow-up was: 73.3% (22/30) in Fu1; 80.0% (24/30) in Fu2; 83.3% (25/30) in Fu3; 76.7% (23/30) in Fu4; 83.3% (25/30) in Fu5; and 73.3% (22/30) in Fu6.
- ✓ The percentage of household members delivering the requested stool sample during the follow-up period ranged between 63.0% (92/146) and 74.6% (94/126).
- ✓ At the household level, only nine children (30%) and at least 50% of their household members were able to deliver a stool sample and receive appropriate treatment in the six follow-ups.

S1 Table. Consort 2010 checklist

CONSORT 2010 checklist of information to include when reporting a randomised trial*

Section/Topic	Item No	Checklist item	Reported on page No
Title and abstract			
	1a	Identification as a randomised trial in the title	1
	1b	Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts)	2
Introduction			
Background and objectives	2a	Scientific background and explanation of rationale	4, 5
	2b	Specific objectives or hypotheses	5, 6
Methods			
Trial design	3a	Description of trial design (such as parallel, factorial) including allocation ratio	6
	3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	N/A
Participants	4a	Eligibility criteria for participants	6, 7
	4b	Settings and locations where the data were collected	5, 6
Interventions	5	The interventions for each group with sufficient details to allow replication, including how and when they were actually administered	7, 8, 9, 10
Outcomes	6a	Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed	11
	6b	Any changes to trial outcomes after the trial commenced, with reasons	N/A
Sample size	7a	How sample size was determined	11 and Supplementary material
	7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomisation:			
Sequence generation	8a	Method used to generate the random allocation sequence	7
	8b	Type of randomisation; details of any restriction (such as blocking and block size)	7,8
Allocation	9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers),	

concealment mechanism		describing any steps taken to conceal the sequence until interventions were assigned	
Implementation	10	Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions	7
Blinding	11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how	N/A
	11b	If relevant, description of the similarity of interventions	8, 9
Statistical methods	12a	Statistical methods used to compare groups for primary and secondary outcomes	11, 12
	12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	
Results			
Participant flow (a diagram is strongly recommended)	13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	12 and Fig 1
	13b	For each group, losses and exclusions after randomisation, together with reasons	15,16 and Fig 1
Recruitment	14a	Dates defining the periods of recruitment and follow-up	6, 12
	14b	Why the trial ended or was stopped	N/A
Baseline data	15	A table showing baseline demographic and clinical characteristics for each group	Table 2 and 3
Numbers analysed	16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	Fig 2
Outcomes and estimation	17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	17, 18
	17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	Supplementary material
Ancillary analyses	18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	Supplementary material
Harms	19	All important harms or unintended effects in each group (for specific guidance see CONSORT for harms)	N/A
Discussion			
Limitations	20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	23, 24
Generalisability	21	Generalisability (external validity, applicability) of the trial findings	24
Interpretation	22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	23, 24, 25
Other information			
Registration	23	Registration number and name of trial registry	3

Protocol	24	Where the full trial protocol can be accessed, if available	http://www.isrctn.com/ISRCTN72928001
Funding	25	Sources of funding and other support (such as supply of drugs), role of funders	Submission system

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

S2 Table. Primary outcomes missing data by study arm

Follow-up (2 years)	Arm 1 (n=29)	Arm 2 (n=31)	Arm 3 (n=31)	Arm 4 (n=30)	Total (n=121)
	n (%)	n (%)	n (%)	n (%)	n (%)
No missing values	24 (82.8)	23 (74.2)	23 (74.2)	26 (86.7)	96 (79.3)
1 missing values	2 (6.9)	4 (12.9)	3 (9.7)	1 (3.3)	10 (8.3)
2 missing values	1 (3.4)	2 (6.5)	1 (3.2)	2 (6.7)	6 (5.0)
3 missing values	1 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.8)
6 missing values	1(3.4)	2 (6.5)	4 (12.9)	1 (3.3)	8 (6.6)

Note: the percentage of primary outcome missings (height, weight, MUAC, and the anthropometric indices of HAZ, WHZ and WAZ) did not differ among the four arms (p=0.534). Little´s test (Chi-Square = 39.952, DF =39, Sig= 0.428) suggested a mechanism MCAR, in terms of missing data

S3 Table. Mean primary outcomes during follow-up by arms and overall

Outcome	Follow-up	A1 (n=29)	A2 (n=31)	A3 (n=31)	A4 (n=30)	*p*-value
		mean ± SD	mean ± SD	mean ± SD	mean ± SD	
Height	**Fu1**	86.61 ± 5.27	88.70 ± 5.84	86.92 ± 5.29	88.02 ± 6.68	0.469
	Fu2	89.38 ± 5.43	91.45 ± 5.82	89.90 ± 4.96	90.67 ± 6.65	0.528
	Fu3	91.95 ± 5.60	94.22 ± 5.50	92.40 ± 5.21	93.39 ± 6.62	0.420
	Fu4	94.48 ± 5.85	96.71 ± 5.35	95.18 ± 4.86	96.01 ± 6.57	0.432*
	Fu5	96.83 ± 5.78	99.23 ± 5.37	97.47 ± 5.01	98.30 ± 6.82	0.408
	Fu6	99.29 ± 5.90	101.35 ± 5.33	99.91 ± 5.09	100.51 ± 6.46	0.548
	Dif fu6-fu1#	12.69 ± 2.01	12.65 ± 1.83	12.99 ± 1.72	12.49 ± 1.65	0.745
Weight	**Fu1**	11.83 ± 1.72	12.39 ± 1.86	12.08 ± 1.48	12.25 ± 1.91	0.572*
	Fu2	12.49 ± 1.86	13.12 ± 1.83	12.88 ± 1.55	13.06 ± 1.94	0.615*
	Fu3	13.20 ± 1.78	13.82 ± 1.84	13.56 ± 1.70	13.66 ± 2.03	0.644*
	Fu4	13.85 ± 1.91	14.45 ± 1.84	14.25 ± 1.65	14.37 ± 2.01	0.580*
	Fu5	14.44 ± 2.13	14.87 ± 1.82	14.75 ± 1.67	14.79 ± 2.20	0.798*
	Fu6	14.67 ± 2.27	15.32 ± 1.92	15.13 ± 1.77	15.36 ± 2.29	0.618*
	Dif fu6-fu1#	2.83 ± 0.95	2.93 ± 0.89	3.05 ± 0.76	3.11 ± 0.73	0.579
HAZ	**Fu1**	-1.33 ± 1.18	-1.29 ± 1.21	-1.33 ± 1.16	**-1.42 ± 1.19**	0.981
	Fu2	-1.31 ± 1.19	-1.23 ± 1.24	-1.25 ± 1.04	-1.38 ± 1.19	0.962
	Fu3	-1.28 ± 1.21	-1.13 ± 1.06	-1.24 ± 1.08	-1.30 ± 1.15	0.941
	Fu4	-1.24 ± 1.22	-1.08 ± 1.00	-1.12 ± 0.99	-1.21 ± 1.12	0.937
	Fu5	-1.19 ± 1.14	-0.99 ± 1.02	-1.11 ± 1.00	-1.19 ± 1.15	0.874
	Fu6	-1.12 ± 1.14	**-0.99 ± 0.98**	-1.05 ± 1.02	-1.15 ± 1.07	0.924
	Dif fu6-fu1#	0.21 ± 0.50	0.30 ± 0.51	0.28 ± 0.38	0.26 ± 0.36	0.849
WHZ	**Fu1**	-0.20 ± 1.09	-0.16 ± 1.14	0.03 ± 0.75	-0.10 ± 0.89	0.793
	Fu2	-0.21 ± 1.04	-0.08 ± 0.96	0.10 ± 0.88	0.05 ± 1.01	0.608
	Fu3	-0.12 ± 0.96	-0.07 ± 1.03	**0.14 ± 0.97**	-0.04 ± 0.98	0.754
	Fu4	-0.11 ± 0.90	-0.07 ± 1.00	0.11 ± 0.94	0.01 ± 1.05	0.829
	Fu5	-0.15 ± 0.94	-0.27 ± 0.76	0.03 ± 0.86	-0.19 ± 0.91	0.579
	Fu6	**-0.51 ± 1.06**	-0.39 ± 0.83	-0.20 ± 0.83	-0.23 ± 0.98	0.542
	Dif fu6-fu1#	-0.31 ± 0.79	-0.22 ± 0.86	-0.23 ± 0.58	-0.13 ± 0.55	0.824
WAZ	**Fu1**	-0.86 ± 1.06	-0.81 ± 1.10	-0.69 ± 0.88	-0.84 ± 0.92	0.909
	Fu2	-0.87 ± 1.06	-0.75 ± 0.96	-0.62 ± 0.86	-0.73 ± 0.92	0.792
	Fu3	-0.80 ± 0.97	-0.69 ± 0.90	-0.60 ± 0.91	-0.76 ± 0.93	0.839
	Fu4	-0.79 ± 0.95	-0.68 ± 0.89	**-0.57 ± 0.85**	-0.70 ± 0.87	0.815
	Fu5	-0.80 ± 1.03	-0.77 ± 0.84	-0.64 ± 0.78	-0.82 ± 0.88	0.845
	Fu6	**-1.00 ± 1.03**	-0.84 ± 0.86	-0.76 ± 0.79	-0.84 ± 0.93	0.752
	Dif fu6-fu1#	-0.15 ± -0.55	-0.03 ± 0.64	-0.07 ± 0.44	0.01 ± 0.31	0.655
MUACZ	**Fu1**	-0.92 ± 1.11	-0.76 ± 1.10	-0.62 ± 0.65	**-0.59 ± 0.82**	0.508
	Fu2	-0.91 ± 0.81	-0.71 ± 0.94	-0.60 ± 0.61	-0.71 ± 0.83	0.518
	Fu3	**-0.95 ± 0.85**	-0.78 ± 0.92	-0.81 ± 0.74	-0.66 ± 0.73	0.591
	Fu4	-0.85 ± 0.81	-0.72 ± 1.02	-0.67 ± 0.59	-0.64 ± 0.67	0.733
	Fu5	-0.87 ± 0.92	-0.80 ± 0.88	-0.64 ± 0.72	-0.71 ± 0.78	0.736
	Fu6	-0.93 ± 0.90	-0.91 ± 0.75	-0.88 ± 0.68	-0.85 ± 0.68	0.977
	Dif fu6-fu1#	-0.01 ±0.75	-0.15 ± 0.89	-0.26 ± 0.67	-0.26 ± 0.52	0.496

From a descriptive point of view, children in A2 were, on average, slightly taller (and older) at entry and persisted with higher mean heights throughout the study. Children in A1 were, on overage, slightly thinner (and younger) at entry and persisted with lower mean weights throughout the study compared to the remaining groups.

Comparing the mean HAZ, WHZ and WAZ from Fu1 to Fu6, no differences were detected among arms. The mean HAZ was slightly higher in A1 compared to the remaining arms. However, after two years of follow-up, slight improvements in mean HAZ were registered mainly in A2, followed by A3, A4 and A1, although mean values remained negative and far from zero in all six moments (ranging from -1.42±1.19 and -0.99±0.98). Considering WHZ, mean values during follow-up were higher compared with HAZ (ranging from -0.51±1.06 and 0.14±0.97), with slight improvements until Fu4, followed by a decreased close to those values observed at the baseline. Mean values of WAZ ranged from -1.00±1.03 to -0.57±0.85 and remained negative and without significant differences throughout the study. Mean values of MUACZ remained negative across follow-up period, ranging from -0.59 ± 0.82 to -0.95 ± 0.85. *Kruskal-Wallis; #difference between initial follow-up (Fu1) and final follow-up (Fu6).

S4 Table. Percentage of stunting, wasting and underweight in children during follow-up and by arms

Variables		Follow-up	A1 (n=29)	A2 (n=31)	A3 (n=31)	A4 (n=30)	*p*-value
			n (%)	n (%)	n (%)	n (%)	
Stunting	**Moderate-to-severe** (HAZ < -2)	**Fu1**	7 (24.1)	11 (35.5)	8 (25.8)	11 (**36.7**)	0.630
		Fu2	7 (24.1)	10 (32.3)	7 (22.6)	10 (33.3)	0.727
		Fu3	8 (27.6)	6 (**19.4**)	9 (29.0)	11 (**36.7**)	0.526
		Fu4	7 (24.1)	7 (22.6)	7 (22.6)	10 (33.3)	0.741
		Fu5	7 (24.1)	6 (19.4)	8 (25.8)	9 (30.0)	0.807
		Fu6	6 (20.7)	6 (19.4)	7 (22.6)	8 (26.7)	0.914
		***P*-value (Fu6 vs Fu1)**§	1.000	0.063	1.000	0.375	
		Dif (%) Fu6 – Fu1#	-3.4	-16.1	-3.2	-10.0	
	Mild-to-severe (HAZ < -1)	**Fu1**	21 (72.4)	21 (67.7)	21 (67.7)	20 (66.7)	0.966
		Fu2	15 (51.7)	20 (64.5)	21 (67.7)	20 (66.7)	0.570
		Fu3	15 (51.7)	17 (54.8)	20 (64.5)	18 (60.0)	0.762
		Fu4	15 (51.7)	16 (51.6)	19 (61.3)	16 (53.3)	0.865
		Fu5	15 (51.7)	17 (54.8)	17 (54.8)	14 (46.7)	0.918
		Fu6	13 (44.8)	15 (48.4)	17 (54.8)	14 (46.7)	0.891
		***P*-value (Fu6 vs Fu1)**§	0.008*	0.031*	0.125	0.031*	
		Dif (%) Fu6 – Fu1#	**-27.6**	**-19.3**	-12.9	**-20.0**	
Wasting	**Moderate-to-severe** (WHZ < -2)	**Fu1**	1 (3.4)	2 (6.5)	0 (0.0)	1 (3.3)	0.652¶
		Fu2	1 (3.4)	0 (0.0)	0 (0.0)	0 (0.0)	0.240¶
		Fu3	1 (3.4)	1 (3.2)	1 (3.2)	2 (6.7)	0.874¶
		Fu4	1 (3.4)	1 (3.2)	1 (3.2)	1 (3.3)	1.000¶
		Fu5	2 (6.9)	2 (6.5)	0 (0.0)	0 (0.0)	0.257
		Fu6	2 (6.9)	0 (0.0)	0 (0.0)	2 (6.7)	0.213
		***P*-value (Fu6 vs Fu1)**§	1.000			1.000	
		Dif (%) Fu6 – Fu1#	3.5	-6.5	0.0	3.4	
	Mild-to-severe (WHZ < -1)	**Fu1**	8 (27.6)	7 (22.6)	1 (3.2)	4 (13.3)	**0.038**¶
		Fu2	7 (24.1)	3 (9.7)	3 (9.7)	5 (16.7)	0.359¶
		Fu3	7 (24.1)	4 (12.9)	2 (6.5)	4 (13.3)	0.282¶
		Fu4	5 (17.2)	4 (12.9)	3 (9.7)	6 (20.0)	0.668¶
		Fu5	6 (20.7)	4 (12.9)	2 (6.5)	8 (26.7)	0.157¶
		Fu6	11 (37.9)	7 (22.6)	3 (9.7)	6 (20.0)	0.079¶
		***P*-value (Fu6 vs Fu1)**§	0.250	1.000	0.500	0.500	
		Dif (%) Fu6 – Fu1#	10.3	0.0	6.5	6.7	
Underweight	**Moderate-to-severe** (WAZ < -2)	**Fu1**	4 (13.8)	4 (12.9)	1 (3.2)	3 (10.0)	0.474
		Fu2	4 (13.8)	3 (9.7)	2 (6.5)	3 (10.0)	0.786
		Fu3	4 (13.8)	1 (3.2)	2 (6.5)	3 (10.0)	0.415
		Fu4	2 (6.9)	3 (9.7)	0 (0.0)	3 (10.0)	0.315
		Fu5	3 (10.3)	2 (6.5)	0 (0.0)	2 (6.7)	0.344
		Fu6	5 (17.2)	2 (6.5)	1 (3.2)	3 (10.0)	0.256
		***P*-value (Fu6 vs Fu1)**§	1.000	1.000	1.000	1.000	
		Dif (%) Fu6 – Fu1#	3.4	-6.4	0.0	0.0	
	Mild-to-severe (WAZ < -1)	**Fu1**	10 (34.5)	13 (41.9)	11 (35.5)	14 (46.7)	0.757
		Fu2	10 (34.5)	12 (38.7)	10 (32.3)	10 (33.3)	0.962
		Fu3	11 (37.9)	12 (38.7)	11 (35.5)	11 (36.7)	1.000
		Fu4	11 (37.9)	10 (32.3)	10 (32.3)	11 (36.7)	0.951
		Fu5	11 (37.9)	15 (48.4)	11 (35.5)	13 (43.3)	0.736
		Fu6	14 (48.3)	14 (45.2)	12 (38.7)	13 (43.3)	0.903
		***P*-value (Fu6 vs Fu1)**§	0.125	1.000	1.000	1.000	
		Dif (%) Fu6 – Fu1#	13.8	3.3	3.2	-3.4	

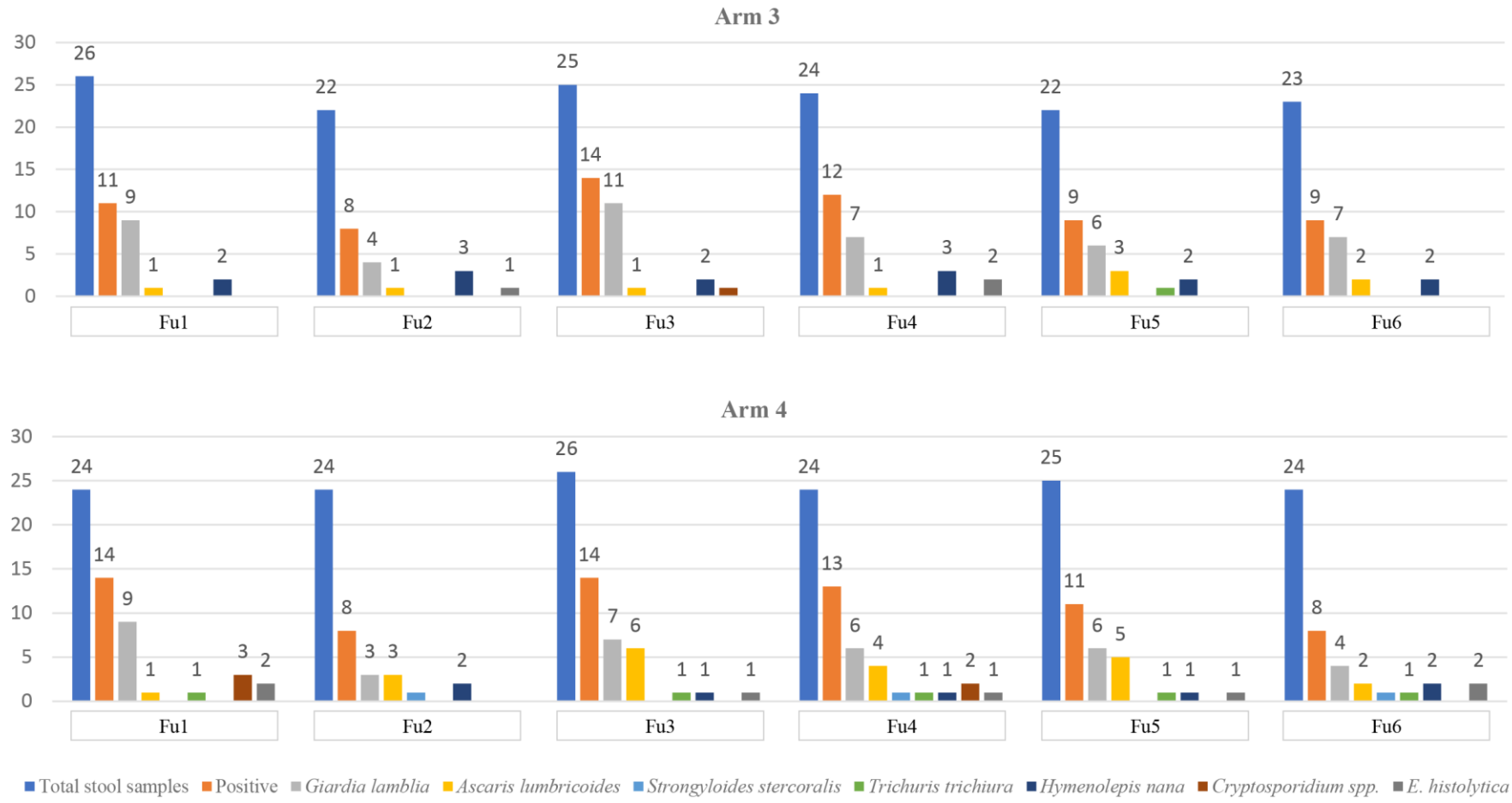
§McNemar´s test; ¶Fisher´s exact-test; #Difference in percentage between initial (Fu1) and final follow-up (Fu6);
**p<0.05*

S5 Table. Percentage of stunting during follow-up and by gender.

Gender	Variable	Follow-up	A1 (n=29)	A2 (n=31)	A3 (n=31)	A4 (n=30)	*p*-value
			n (%)	n (%)	n (%)	n (%)	
Female (N=61)	**Moderate-to-severe stunting** (HAZ < -2)	**Fu1**	3 (23.1)	6 (46.2)	4 (22.2)	5 (29.4)	0.540
		Fu2	3 (23.1)	6 (46.2)	3 (16.7)	3 (17.6)	0.266
		Fu3	4 (30.8)	3 (23.1)	4 (22.2)	4 (23.5)	0.954
		Fu4	3 (23.1)	4 (30.8)	2 (11.1)	3 (17.6)	0.597
		Fu5	3 (23.1)	3 (23.1)	3 (16.7)	3 (17.6)	0.947
		Fu6	2 (15.4)	3 (23.1)	3 (16.7)	2 (11.8)	0.934
		***P*-value* (Fu1 vs Fu6)**	1.000	0.250	1.000	0.250	
	Mild-to-severe stunting (HAZ < -1)	**Fu1**	10 (76.9)	9 (69.2)	12 (66.7)	11 (64.7)	0.929
		Fu2	8 (61.5)	9 (69.2)	11 (61.1)	11 (64.7)	0.983
		Fu3	9 (69.2)	8 (61.5)	11 (61.1)	9 (52.9)	0.847
		Fu4	9 (69.2)	9 (69.2)	11 (61.1)	7 (41.2)	0.357
		Fu5	9 (69.2)	9 (69.2)	10 (55.6)	7 (41.2)	0.359
		Fu6	7 (53.8)	8 (61.5)	10 (55.6)	6 (35.3)	0.532
		***P*-value* (Fu1 vs Fu6)**	0.250	1.000	0.500	0.063	
Male (N=60)	**Moderate-to-severe stunting** (HAZ < -2)	**Fu1**	4 (25.0)	5 (27.8)	4 (30.8)	6 (46.2)	0.283
		Fu2	4 (25.0)	4 (22.2)	4 (30.8)	7 (53.8)	0.160
		Fu3	4 (25.0)	3 (16.7)	5 (38.5)	7 (53.8)	0.160
		Fu4	4 (25.0)	3 (16.7)	5 (38.5)	7 (53.8)	0.160
		Fu5	4 (25.0)	3 (16.7)	5 (38.5)	6 (46.2)	0.299
		Fu6	4 (25.0)	3 (16.7)	4 (30.8)	6 (46.2)	0.359
		***P*-value* (Fu1 vs Fu6)**	1.000	0.500	1.000	1.000	
	Mild-to-severe stunting (HAZ < -1)	**Fu1**	11 (68.8)	12 (66.7)	9 (69.2)	9 (69-2)	1.000
		Fu2	7 (43.8)	11 (61.1)	10 (76.9)	9 (69.2)	0.315
		Fu3	6 (37.5)	9 (50.0)	9 (69.2)	9 (69.2)	0.261
		Fu4	6 (37.5)	7 (38.9)	8 (61.5)	9 (69.2)	0.219
		Fu5	6 (37.5)	8 (44.4)	7 (53.8)	7 (53.8)	0.757
		Fu6	6 (37.5)	7 (38.9)	7 (53.8)	8 (61.5)	0.515
		***P*-value* (Fu1 vs Fu6)**	0.063	0.063	0.500	1.000	

* McNemar´s test

S1 Fig. Infection with pathogenic intestinal parasites in children during follow-up: A3 and A4


Arm 3
30
25
20
15
10
5
0
26 11 9 1 2
22 8 4 1 3 1
25 14 11 1 2
24 12 7 1 3 2
22 9 6 3 2
23 9 7 2 2
Fu1
Fu2
Fu3
Fu4
Fu5
Fu6
Arm 4
30
25
20
15
10
5
0
24 14 9 1 1 3 2
24 8 3 3 2
26 14 7 6 1 1 1
24 13 6 4 1 1 2 1
25 11 6 5 1 1 1
24 8 4 2 1 2 2
Fu1
Fu2
Fu3
Fu4
Fu5
Fu6
Total stool samples
Positive
Giardia lamblia
Ascaris lumbricoides
Strongyloides stercoralis
Trichuris trichiura
Hymenolepis nana
Cryptosporidium spp.
E. histolytica

3. DISCUSSION AND CONCLUSIONS

Similar to other low- and middle-income countries, in Angola, malnutrition and diarrhoeal disease are among the major causes of deaths in children under-five years of age (1-3). Data related to malnutrition prevalences from the Multiple Indicators Survey in 2015-2016 indicates that 37.6% of children are suffering from stunting, 4.9% from wasting, and 19.0% from underweight (4). As previously mentioned in the introduction section of this thesis, the national nutrition goal is to reduce stunting to less than 5%, wasting to less than 5% and underweight to less than 10% by 2021 (2). Current studies focusing on malnutrition and enteric infections in the paediatric population of Angola are still scarce (5-12).

In this thesis two studies were performed: one hospital-based cross-sectional study aiming to identify the aetiological agents of diarrhoea in 344 children under-five years attending the Bengo General Hospital between September 2012 and December 2013, including the molecular characterization of rotavirus circulating genotypes and *Giardia lamblia* assemblages and subassemblages; and a four-arm randomised parallel trial during two years to investigate if treatment of intestinal parasites (with or without previous diagnosis) in two different levels (individual or household) impacts on nutritional status of 121 children between 2 and 5 years old. Main findings of both studies are summarized in Annexe IV.

High levels of malnutrition were found among children included in both studies (cross-sectional and longitudinal): moderate-to-severe stunting (32.4% and 30.6%), wasting (31.5% and 7.5%) and underweight (34.5% and 16.0%). However, a great proportion of children were suffering from mild malnutrition in both studies: mild stunting (22.2% and 33.1%), mild wasting (19.5% and 33.1%) and mild underweight (26.6% and 29.4%), showing the importance of intervening in a timely manner in this group of children to prevent worsening of the nutritional status. The discrepancies between two studies are expected, since the first one included under-five children with diarrhoea (of which more than 80% were under 24 months of age); and the second study included older children (aged 20-36 at the recruitment to be followed-up between their second and fifth year of

life) infected with at least one pathogenic intestinal parasite, regardless presenting diarrhoea or not.

Considering the living conditions, the results were also similar between the cross-sectional and the longitudinal studies: near 20% of children did not have access to a latrine (in both studies); the drinking water sources more frequently used were the river (27.0% in the first study and 24.8% in the second study), the tap in the yard (24.4% and 27.4%), the private tank (24.4% and 29.1%) and the public tap water (13.3% and 7.7%); and among those treating water before consuming (60.2% and 42.7%), bleach (82.1% and 88.0%) and boiling were the most frequent methods applied. These results show that a large proportion of children does not have access to safe water, hygiene and sanitation conditions. Indeed, the majority of Angolans, even in peri-urban areas, are still depending on informal mechanisms for water supply, most of them of poor quality and, thus, representing a significant risk for the health (13).

In the cross-sectional study, it was found that 66.0% of children were infected with at least one pathogenic enteric agent. Most cases of diarrhoea were due to *Cryptosporidium* (30.0%), rotavirus (25.1%) and *Giardia lamblia* (21.6%). Other enteric pathogens were also identified, such as *diarrheagenic Escherichia coli* (6.3%), *Ascaris lumbricoides* (4.1%), adenovirus (3.8%), *Strongyloides stercoralis* (3.5%), astrovirus (2.6%), *Hymenolepis nana* (1.7%), *Entamoeba histolytica/díspar* (0.9%), *Taenia* spp. (0.6%), *Trichuris trichiura* (0.3%), and *Entamoeba histolytica* (0.3%). Twenty-five percent of children had multiple infections. These results are important to national health policies, since the identification of the agent causing diarrhoea is essential to define better strategies related to the primary and secondary health sectors (e.g., vaccination and treatment regimens, respectively).

In this context, before the introduction of rotarix vaccine in the national immunization plan, the Angolan Ministry of Health sponsored a research study to provide baseline information on the molecular profile of rotavirus (5). It was a cross-sectional study addressing viral agents (first rotavirus, and then norovirus and astrovirus) carried out in health units, mainly located in urban areas of four provinces: Huambo (June 2012), Zaire (July-August 2012), Luanda (September-October 2013) and Cabinda (September-October 2013). Infection rates varied between the provinces: Zaire (56.0%), Huambo

(37.4%), Luanda (22.7%) and Cabinda (14.6%), and a globally common G1P[8] was found (50.0%), along with the uncommon strain G1P[6] (29.3%) and G2P[4] (5.2%) (5).

The study conducted in Bengo, also before the vaccine introduction, contributed with new information since there was no previous data on the burden of rotavirus for this province, nor during a year-period in the country. It was found that 25.1% of children were infected, and similar to the previous study, G1P[8] was the most frequent genotype (47.2%), followed by G1P[6] (29.2%) and G2P[4] (12.5%). A systematic review investigating the prevalence of RVA genotypes circulating in Africa between 2006 and 2016 found that G1P[8] was also the most encountered combination (22.6%), followed by G2P[4] (8.3%), G9P[8] (7.0%) and G2P[6] (5.0%) (14), though difference in circulating strains were found across years, within regions and countries (14).

Associations between rotavirus infection and other sociodemographic variables were also explored in the cross-sectional study. Rotavirus infection rate was more frequent in children less than 12 months, presenting vomiting, and attending the emergency service unit. No significant association was found between rotavirus infection and the type of water source or sanitation conditions, probably due to the fact that rotavirus is ubiquitous, and it is not confined only in settings with poor hygiene and sanitation conditions (15). Thus, since rotavirus is highly contagious (faecal oral and person-to-person transmission, as well through fomites in the environment), and without an effective treatment such as antibiotics, severe cases can rapidly deteriorate the nutritional status of children, leading to severe dehydration and death (16). This enhances the importance of rotavirus vaccine introduction in the country in order to prevent morbidity and mortality, especially in infants, along with other strategies, such as access to adequate WASH and early rehydration therapy (1, 15). Thus, the introduction of the vaccine in Angola was recognized as a priority, and it was formalized in April 2014 with Rotarix vaccine to be administered at two and four months of age. This marked two distinct eras: pre- and post-introduction of the vaccine.

According to WHO Surveillance Standards of Vaccine-Preventable Diseases, during the pre-era, countries must generate information to facilitate and support the introduction of rotavirus vaccine, related to the epidemiology and burden of associated hospitalizations, clinical presentations and outcomes of the disease, seasonality, and the prevalence of

circulating strains (17). The collection of data in at least one sentinel site per country, among children under-five, and preferably by including 250-500 cases during two full years prior to vaccine introduction, is also recommended (17). Thus, considering these guidelines, it is undeniable the contribution of the study conducted in Hospital Geral do Bengo, although not performing collection data during the full two year-period. Findings from this research were formally communicated to the Ministry of Health, and disseminated through publication in international journals, presentations in national and international conferences, television, radio and national health newspaper.

Since the rotarix introduction in Angola, according to the update estimates reported by WHO (21 September 2018), coverage has been increasing over the years, achieving in 2017 a rate of 82% in the first dose administered and 68% in the second dose (18). As emphasized in October 2018, during the Meeting of the Strategic Advisory Group of Experts on Immunization - SAGE (October, 2018), it is essential to perform surveillance and obtain data at national and subnational levels after the vaccine introduction (19). Rotavirus vaccination has showed to substantially reduce global deaths of children younger than five years, which is a target included in the Sustainable Development Goals (20). Therefore, it is expected that the introduction of rotarix vaccine will be likely to contribute to the reduction of infant and under-five mortality rates in Angola, which in turn can lead to better health and socioeconomic outcomes of the country (20). However, gains must be monitored and maintained. A recent systematic review with post-licensure data from 24 countries has found a median Rotarix™ effectiveness of 84%, 75%, and 57% in countries with low, medium, and high child mortality, respectively (152). Moreover, Rotarix™ vaccine was recently associated with substantial reduction in diarrhoea-associated deaths among infants in a rural community of Malawi (34%), showing the important impact of rotavirus vaccine programs (21). In the European & Developing Countries Clinical Trials Partnership (EDCTP) stakeholder meeting on diarrhoeal diseases (July, 2016), rotavirus prevention was not identified as a high priority area for short-term research since the vaccine is providing good results, but it was enhanced that vaccine effectiveness needs continued monitoring to certificate that vaccine covers the diversity of strains in SSA (22). Until this moment, no research studies were published addressing the impact and effectiveness of rotavirus vaccine in reducing diarrhoea-associated mortality in Angolan infants. Research studies conducted during the

pre-era vaccination will be crucial as baseline information to compare with pos-era vaccination studies.

The first study of this thesis added relevant information on the burden of enteric disease, addressing not only viruses, but also parasitic and bacteria infections in under-five children. Regarding bacterial agents, *Escherichia coli* was confirmed to be pathogenic in 19 of 97 isolates: 12.4% EAEC and 7.2% ETEC. A multisite birth cohort study (MAL-ED), conducted between 2009 and 2012 in eight sites in South America, Africa, and Asia have identified *Campylobacter* spp. as the enteric agent with the highest attributable burden of diarrhoea during the first two years, as well as *Shigella*, though only during the second year (23). After applying molecular procedures in the same samples, *Shigella* spp., *Escherichia coli*, *Campylobacter* spp. and typical enteropathogenic *Escherichia coli* continued to be among the top ten pathogens accounted for attributable diarrhoea (24).

Conversely, *Campylobacter jejuni*, *Shigella* spp and *Salmonella* spp. were not isolated in this study. In poor-resource settings performing conventional microbiologic techniques can be challenging due to the constant problems related to the electrical power, which can interfere with the laboratory procedures (25, 26). Thus, our bacterial results should be interpreted with caution, and future studies are needed to better address the prevalence of these infections among preschool children in Angola. Indeed, there are cultural habits in the country that can influence the faecal-oral transmission of pathogens. For instance, similar to other low and middle-income countries, the informal street food vending is common in Angola, and is usually conducted outside the regulation of national laws. There is evidence that the preparation of street foods with inadequate hygienic conditions, the time that the food remains at ambient temperature, and serving with bare hands contribute to the transmission of pathogens of public health importance, mainly *Campylobacter jejuni*, *Salmonella* spp. and *Shigella* spp. However, due to the relatively inexpensive prices, food street attracts mainly, but not only, individuals with lower purchasing power (27, 28).

The lack of hygiene conditions is an important factor also among intestinal parasites transmission. For instance, in the MICS survey 2015-2016, 11% of caregivers reported giving other food or liquid than breastmilk in the first three days of the child´s life (4). Considering that a great proportion of caregivers reported drinking water from river or

from the tap in the yard without any previous treatment, children can became easily infected even in the first months of their lives, as shown in the study addressing the aetiological agents of diarrhoea. In the same study, helminthic infections were more common in children older than 12 months. At this age, children start walking barefoot, playing in contaminated soils and putting their hands in the mouths. Thus, these environmental conditions where children spend their time can increase the risk for infections. Furthermore, the high temperature of this region can also influence the dehydration of children who spend considerable time away from home without supervision of caregivers (29).

In the cross-sectional study, *Cryptosporidium* spp. was the most common pathogenic identified, especially during infancy. More recently, a study conducted in Cubal, Benguela province, have also reported *Cryptosporidium* spp. infection after molecular analysis of stool samples of school-aged children (2.9%) (10). Results from Bengo study are in accordance to findings from MAL-ED study, where *Cryptosporidium* spp. was the second leading agent, after rotavirus, causing the highest burden of diarrhoea in children less than 12 months (23). Moreover, this protozoan has been also associated in developing countries with a greater than two-fold increase in mortality in children aged 12-23 months with moderate-to-severe diarrhoea (30).

These findings highlight the importance of *Cryptosporidium* infection, and considering the results from Bengo province, more attention should be given to the burden of the disease among infants. Oocysts of *Cryptosporidium* are stable and persistent in the environment, and can be found in contaminated water sources (31-33). In this study, no significant association was found between the type of water source used and this infection. As previously reported, the use of unsafe water for drinking is common, and when treated, bleach was the most common treatment method applied. We did not collect data to understand if the treatment method was correctly applied. However, when talking to the caregivers it was possible to perceive that a great proportion did not applied it correctly (e.g. inadequate number of bleach drops in relation to the amount of water to be treated, or boiling during few minutes, less than the necessary). Despite this, it is known that water disinfection with chlorine is not effective against *Cryptosporidium* spp., an important waterborne protozoan parasite (34), and boiling was a method applied by small proportion of caregivers, which enhances the need for safe drinking water source (35).

Regardless of the method of water treatment, it was possible to understand that providing information on the adequate treatment of drinking water is essential. Findings from a recent systematic review also did not report a significant association between poor drinking water quality and *Cryptosporidium* spp. infection in low- and middle income countries (33). In our cross-sectional study no association was found between *Cryptosporidium* spp. infection and sanitation conditions. However, according to the systematic review previously referred, lack of appropriate sanitation/ open defecation (OR: 1.82; 95%CI: 1.19-2.8), animal contact (OR: 1.98, 95%CI: 1.11-3.54), diarrhoea in the household (OR:1.98, 95%CI: 1.13-3.49), and overcrowded living conditions (OR:1.37, 95%CI: 1.07-1.75) were also important risk factors for the infection (33).

Our cross-sectional study found a higher proportion of wasting in children infected with *Cryptosporidium* spp. compared to those not infected (34.1 versus 25.8%), only significant in the univariate analysis. Infection with *Cryptosporidium* spp. has been previously associated with malnutrition, stunted growth and cognitive impairment (31). A longitudinal birth cohort study conducted in Bangladesh found that 77.0% of children experienced at least one infection with *Cryptosporidium* spp. in the first two years of life (36), and that extreme poverty was associated with higher rates of infection, leading to a greater than two-fold increased risk of severe stunting at age two, compared to those not infected (36).

Regarding treatment strategies, nitazoxanide is the only available drug for treatment in children older than 12 months, and it was previously reported an efficacy ranging from 56% to 96% in healthy individuals (32). However, it is less effective in immunocompromised individuals, such as those HIV positive and/or malnourished (32, 37-40). Thus, *Cryptosporidium* spp. infection is a great challenge, especially in settings with poor sanitation and in HIV-positive individuals (32, 36, 38, 41). The need for more effective drugs was defined as a high priority level in the EDCTP meeting on diarrhoeal diseases in 2016, and it was also suggested conducting a clinical trial to investigate the effect of the drug on stunting (22). Until better treatment strategies are found, a great investment should be done to increase the access to safe drinking water and adequate sanitation conditions (33).

The cross-sectional study included in this thesis found a large proportion of children with diarrhoea infected with *Giardia lamblia* (21.6%). Other studies conducted in Angola also reported high infection rates, but in school-age children, in the provinces of Bié (18.0%) Huila (20.1%) and Benguela (37.9%) (7, 8, 10). Additionally, findings from Bengo study have also provided a molecular characterization of *Giardia lamblia*, showing a predominance of assemblage B among children with acute diarrhoea (11/12, 91.7%), while assemblage A was identified in only one child (1/12, 8.3%). Despite the few samples used, this was the first report providing information on the molecular profile of this intestinal protozoan. More recently, a higher frequency of *Giardia* assemblage B (18/28, 64.3%) compared with assemblage A (10/28, 35.7%) was also reported in Cubal, Benguela (10). In Rwanda, a study conducted in a rural community (84.4%) and health units (15.6%) showed that, off all intestinal parasites, *Giardia lamblia* was the most frequent in under-five children, with a prevalence of 19.8% considering microscopic diagnosis, and 60.1% after molecular analysis with PCR (42).

In our longitudinal study, a great proportion of *Giardia lamblia* was found in asymptomatic children compared with children with diarrhoea, at baseline. From a public health perspective this is an important issue, since asymptomatic children can shed cysts, and thence contribute for the transmission of disease. This highlights the need to address giardiasis among children, in parallel with soil-transmitted helminth infections. However, only STHs are targeted in the regular preventive chemotherapy (deworming) recommended by WHO (43).

According to the most recent Cochrane systematic review, in communities where intestinal helminths are endemic, the effect of a single dose deworming drug probably has little or no effect on average weight gain (moderate quality evidence), on average haemoglobin (moderate quality evidence), and on cognition (low quality evidence) of children (44). Similarly, according to another systematic review, including 52 studies from low- and middle-income countries, the effect of mass deworming for STHs was also reported to have little effect on growth and other health outcomes of children between 6 months and 16 years (45). A cluster-randomised trial including one million pre-school children in north India has also shown little or no effect of regular deworming on mortality (46). Conversely, in a research study using data from 45 Demographic and Health Surveys, it was recently reported that deworming was consistently associated with

stunting and anaemia reduction in SSA and, thus, supporting the benefits of deworming among preschool age children (47). Results are controversial and the benefits of deworming on nutritional status of children are still under discussion (48).

To our knowledge, the four-arm randomised controlled trial included in this thesis was the first longitudinal study performed in Angola to investigate the effect of different treatment strategies (annual albendazole versus screening and treating intestinal parasites) at two levels (individual and household) on nutritional status of children aged 2-5 years. Some results of this study were summarized in the fourth paper, but additional data can be explored in the future.

Briefly, 121 children infected with at least one pathogenic intestinal parasite were included for two years of community follow-up. A sociodemographic survey applied in this study also provided new information regarding the household environment. For instance, household material construction had mainly adobe walls (74.4%), cement or ceramic floor (87.2%) and iron sheets roof (95.7%), as illustrated in Annexe V – Photos from fieldwork. The majority of assets had at least a mobilephone (94.0%), television (88.0%), public electricity (80.3%). Freezers (75.2%) were more commonly used than generators (23.9%) or refrigerators (17.9%). After an exploratory analysis, we have found that the proportion of stunting was higher in houses with adobe walls compared with those made of bricks, and in houses with earth or sand floors compared with cement floor, enhancing the contribution of poor living conditions in the health of children.

Findings from the longitudinal study suggest that screening and treating intestinal parasites did not result in better growth outcomes compared to annual albendazole, regardless it was performed at individual or household level. According to previous research, the benefits of deworming is likely greatest among children with severe intensity of infection (43). However, at baseline of our longitudinal study children were lightly infected with STH, which could have also contributed to these results. Besides, deworming does not interrupt the cycle of transmission, and without changing the evolving environmental conditions, they remain vulnerable to reinfections (49).

Besides, there are other nutrition-factors that were not addressed in this thesis but could have contributed for this result: the diet quality (which can be influenced by factors related to the consumer, such as time, knowledge, purchasing power, and preferences)

and the food environment (related to food production, crop yields, price, quality and physical access) (50, 51). In Angola, all these factors were recently recognized as determinants for better nutritional outcomes during the first International Conference on Food and Nutrition Security that took place in Luanda, the capital province. Promoting agricultural production, ensuring food storage and transport, transformation and retailing is essential. Agriculture sector is now a priority area for action in Angola given its expected impact on employment growth, household income and, consequently, in reducing poverty (52).

To assess the evolution of nutritional status, in our longitudinal study anthropometric indices were calculated at baseline and every four months until the end of the study. At baseline, the overall mean observed for anthropometric indices were: HAZ -1.34±1.33; WHZ -0.29±1.18; and WAZ -0.80±0.98. After two years, the mean Z-scores values remained negative for all anthropometric indices: HAZ -1.08±1.04, WHZ -0.33±0.92, and WAZ -0.86±0.90. Similar to our baseline results, mean Z-scores of children under-five years included in the MICS 2015-2016 were: HAZ -1.5; WHZ 0.1; and WAZ -1.0 (4), showing this is an important issue to be addressed not only in Bengo province, but also at national level. Moreover, compared to other regions of the world, SSA is the region with the shortest children, after South Asia, while the tallest are in Europe and Central Asia, almost to 1.5 Z-scores higher than the obtained for SSA region, for children between two and five years (53).

An important factor that can influence child malnutrition is related to maternal health, also not addressed in this thesis. Growth faltering has been reported mainly in low- and middle-income countries, where children are already born with mean values below the WHO reference and continue to decrease substantially until two years of age, enhancing the importance of interventions targeting the first 1,000 days of life (including pregnancy and the first two years of the child´s life), also known as *window of opportunity* for preventing undernutrition (53).

Although there was no evidence of significant effect on nutritional outcomes by any of the treatment strategies performed in the longitudinal study, we have found an important reduction of mild malnutrition, even beyond the *window of opportunity*. It is true that severe malnutrition poses an increased risk of death in children (54, 55). However,

children with mild malnutrition are vulnerable to variations of their nutritional condition. Although Angola has been at peace for more than a decade, there are regions where children are vulnerable to poverty, which can contribute to worsen their nutritional status. Thus, greater attention should be given to this neglected degree of malnutrition.

Regarding the interventions provided in arms 3 (screening and treating at individual level) and 4 (screening and treating at household level), it was possible to observe, from the initial to the final follow-up, a significant reduction of *Giardia lamblia* infection among children from arm 4, but not among children of arm 3, enhancing the importance of transmission among individuals living in the same household. Given the great dimension of data collected in the longitudinal study, pathogenic intestinal parasites identified among the household members are not presented in this work, but it is our intention to address this subject in the near future.

Implementing a longitudinal study in a poor setting is challenging, and not surprisingly, our sample size was slightly smaller than the theoretical sample calculated. This was due to a high proportion of children not delivering the stool sample at the recruitment (compared to what we have expected), as well as those children that met the inclusion criteria but did not appear for treatment before allocation. In this longitudinal study, we not only included 121 children, but also household members: for example, in Arm 2 the single dose of albendazole was also given to 125 household members in the first follow-up (83.3% of the total, 125/150), and 123 in the fourth follow-up (80.9% of the total, 123/152). Moreover, in Arm 4, a stool sample was requested to each one of the members living in the same household in all follow-ups. Logistically this becomes much more demanding because families are monitored over time and the success of interventions requires not only access to the child, but also the collaboration of all household members.

It is important to keep in mind that a follow-up visit interferes with the dynamics of each member of the family, their leisure time, work schedule and their responsibilities to the community (56). For these and other reasons, it is extremely important to be aware of the social context of the study and the importance of promoting good communication and flexibility in order to enhance the participation of all members of each household included. In this study, we included only participants with residence in the Dande HDSS study area, since a geographical coordinate is generated for each household. However,

even with this great support, during community- follow-up, sometimes it was difficult to access the houses of the participants for different reasons: poor condition of roads[11], some streets have no name, and reference available to vague outstanding points (e.g. near the old tree, by the river, next to the house of the hamlet coordinator). Some of these difficulties were addressed by including the support of local collaborators. Even so, 79% of children were successfully followed-up in the community for the six assessments scheduled during the study.

In terms of public health, these findings suggest that to improve children nutritional status, deworming alone or other treatment strategies might not be sufficient, and probably longer interventions would be necessary to decrease height deficits in children. This was the first longitudinal trial contributing with new research approaches by emphasizing the importance of including intestinal protozoa, in addition to helminthiasis, when thinking on therapeutic interventions to improve the growth of preschool children. Moreover, growth patterns using repeated measurements in six time points were assessed during the follow-up. The interventions were carried-out considering individual and household level, which should be also addressed in future studies given the dynamics of transmission of most pathogenic agents.

3.1 Limitations and strengths

Limitations of the studies conducted were already mentioned in detail in each one of the papers presented in the results section of this thesis. The first thing that is important to mention is that the study's results cannot be generalized to the entire population given its design concept. The second aspect is related to the inclusion criteria. For instance, in the cross-sectional study, only children with diarrhoea were included. However, asymptomatic children can also serve as reservoirs of infections, ensuring transmission to other individuals at community level, and they were not included in the cross-sectional study given the fact that we aimed to address the aetiology of diarrhoea. In the case of rotavirus, probably the infection rate was underestimated since we have not included

[11] See Annexe V – Photos from field work

children presenting only vomiting, and it is known that rotaviruses are shed for several days in very high concentrations in the stools and vomitus of infected individuals (15).

There are also laboratory diagnosis limitations important to mention here. In the cross-sectional study, only one stool sample per child was requested, which could have underestimate the rate of infection given the intermittent shedding of some intestinal parasites (57). Similarly, in the longitudinal study we have also requested only one stool sample per follow-up assessment. Requiring three instead one sample in each moment would be logistically more difficult for caregivers, and thus, could contribute to increase losses to follow-up. Moreover, since we were dependent on the delivery of the stool sample, we cannot guarantee that it was done right after of defecation, and this could have influenced microscopic diagnosis of pathogenic agents such as hookworm (eggs degrade rapidly) and *Giardia lamblia* (in diarrhoeal stools, trophozoites disappear rapidly) (57, 58). Microscopic detection of *Strongyloides stercoralis* could have been underestimated given the low sensitivity of techniques performed (59). Regarding molecular procedures applied in the cross-sectional study: for characterization of rotavirus genotypes, we found some barriers related to the reduced amount of diarrhoeal samples; and for molecular characterization of *Giardia lamblia*, only in few samples DNA was successfully sequenced, which could have been due to DNA degradation.

Another limitation is related to medication regimens in the longitudinal study. According to the study treatment protocol, some treatments included more than one dosage/days of treatment, and we cannot guarantee that caregivers have complied with the prescription at home. Self-reported data from questionnaires are also prone to social desirability response bias (a limitation difficult to overcome in any study).

The strengths of our cross-sectional study include the identification of the main causes of diarrhoea in children under-five living in Bengo, as well as associated risk factors, during a year-period. This is important, so interventions can be conceptualized based on realistic information and targeting the age groups of children at higher risk of enteric infections. The identification of rotavirus strains was very important since it was performed before the vaccine introduction and it will be essential for comparison in future studies.

Considering the longitudinal study, this trial included seven repeated measurements from the same participant (including baseline), which is a key strength of studies with this type

of design. A study with repeated measurements allows to chart the profile of the same individual across time, which is an advantage compared with cross-sectional study, where different subjects are commonly observed at different times (60). Adherence to interventions differed across arms of the study and this may have contributed to bias. This limitation is more concerned in a complete analysis. However, to overcome this limitation, we performed an intention-to-treat analysis, according to CONSORT guidelines, after a missing data analysis (61). Missing values can represent a huge challenge in longitudinal studies (62). In this study, different methods were applied, such as: interpolation for height values and multiple imputation using the Expectation Maximization for other measurements (62). On the other hand, data collected from a longitudinal study is more reliably that would be data from cross-sectional study, given the fact that the same children were followed repeatedly, and it decreased the risk of collecting incorrect anthropometric data because the height does not decrease and subjects can serve as their own controls (60). Moreover, since we have focused on the effect of treatment strategies on nutritional status of each one of the participants included over time, smaller sample sizes are needed compared with cross-sectional designs (60).

3.2 Conclusions

After suffering a long-term period of civil war, Angola has made a huge progress in the socioeconomic and health indicators, but there are still improvements to be made. In Bengo province, children are exposed to a wide variety of enteric pathogenic agents in the first years of life. Findings from studies conducted under this thesis confirmed that children under 12 months are at higher risk of rotavirus and *Cryptosporidium* spp. infections, which have been identified as one of the major causes of moderate-to-severe diarrhoea contributing to malnutrition and death in developing countries. In addition, older children are also at risk of acquiring other infections caused by soil-transmitted helminths, which can affect the nutritional status of children. In terms of primary prevention, a major achievement was the introduction of the rotavirus vaccine in the national immunization plan in 2014. In the future, efforts must be made to assess the effectiveness of the rotarix vaccine in the country. For the control of soil-transmitted helminths, deworming is a public health intervention recommended by WHO, also adopted in Angola. At the moment, the benefits of deworming on the nutritional status of children are under discussion in the international research community. The two-year

longitudinal study performed in this thesis did not find differences between giving albendazole once a year or diagnosing and treating intestinal parasites, both at individual and household levels, in the nutritional status of children 2-5 years. Additional research is needed to address the benefits of deworming in the nutritional status of children in more heavily infected children, and investigate the dynamics of infection between the different pathogenic agents.

In both studies performed in this thesis, more than 30% of children were suffering from stunting. After, in 2016, national data registered 38% of stunting in Angola and 40% in Bengo province (MICS 2015-2016). The national goal of reducing stunting to less than 5% by 2021 seems very ambitious and unachievable. However, efforts must not stop during the sustainable development era. Decision-makers must be aware of socioeconomic consequences of malnutrition not only in the present, as well in the future generation, for a stronger political commitment. Social and financial investments are essential to improve people's living conditions, especially in rural areas, where they face a higher risk of poverty. Malnutrition and inadequate development of children are a result of multiple factors. Therefore, a multidisciplinary approach within different governmental sectors is required to improve access to safe water, adequate sanitation and hygiene conditions, and access to health care services. In addition, education and agriculture are also key sectors, in order to increase food production and availability. Thus, each child should be able to access foods with nutritional value and according to his/her health status and age.

3.3 Recommendations

Scientific research is essential for policy decision-making, however, it is still scarce in Angola. A greater recognition of the importance of research in decision-making is necessary, and, on the other hand, it is essential that research studies manage to focus on the major health problems, giving particular attention to children as “future adults”.

Improving laboratorial capacity for diarrhoeal diseases is needed for a better and more timely treatment and also for more rigorous epidemiological studies (22, 25). Since Angola is now in the pos-era of the vaccine introduction, rotavirus surveillance is important to support national vaccination strategies (17, 19). In this context, it is

recommended to monitor the impact of rotavirus vaccination on disease reduction (requires laboratory capacity of health units to perform routine diagnosis through enzyme immunoassays for antigen detection) and monitor the changes in the circulating strains (using more complex techniques such as reverse transcription polymerase chain reaction) (15). After interacting with laboratory technicians from Bengo, it was possible to verify that stool sample were collected only for the microscopic detection of intestinal parasites, through direct examination. However, improvements must be made not only in the preparation of the stool sample, but also in the ability to identify intestinal protozoa and helminths. Intestinal protozoa such as *Giardia lamblia* are completely neglected and, thus, undiagnosed. Strengthening human resources capacity and training is essential for identifying the aetiological agents of infection. This is important to provide adequate treatment and avoids the prescription of antibiotics in cases where it is not indicated (25).

In our study, we included mainly children from urban areas. However, given disparities within regions, special attention should be given to children living in rural areas, where poverty limits the access to adequate food, adequate health care services, safe drinking water source, and sanitation conditions. Actions to promote early breastfeeding should be adopted and its importance transmitted to caregivers in both hospital and community settings. Nutrition education at community level should also be performed so caregivers are able to identify, among the available food, those more adequate for the child´s age. Moreover, it is also important to inform the population on how to correctly treat the water before drinking. In this context, in the final 2014, a cluster randomised trial was started in the Health Research Center of Angola (CISA), Bengo province, to compare the efficacy of two community-based interventions (nutritional education vs wash, sanitation and hygiene practices, both with additional treatment strategies of infectious diseases including malaria, schistosomiasis and soil-transmitted helminths) in the reduction of anaemia in preschool children (63). The results obtained from this study (not published yet) can lead to important contributions in this field. In settings such as Bengo province, the radio and the church are one of the most important means for the dissemination of information and, therefore, should be used more routinely to educate the population.

3.4 Discussion and conclusion references

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ANNEXES

Annexe I - List of the 17 Sustainable Development Goals

ANNEXES

Annexe I – List of the 17 Sustainable Development Goals

The Sustainable Development Agenda includes 17 Goals (Figure 20) and 169 targets to be achieved by 2030.

Sustainable Development Goals (SDGs)			
1 NO POVERTY	End Poverty in all its forms everywhere	10 REDUCED INEQUALITIES	Reduce inequality within and among countries
2 ZERO HUNGER	End Hunger, achieve food security and improved nutrition and promote sustainable agriculture	11 SUSTAINABLE CITIES AND COMMUNITIES	Make cities and human settlements inclusive, safe, resilient and sustainable
3 GOOD HEALTH AND WELL-BEING	Ensure healthy lives and promote well-being for all at all ages	12 RESPONSIBLE CONSUMPTION AND PRODUCTION	Ensure sustainable consumption and production patterns
4 QUALITY EDUCATION	Ensure inclusive and equitable quality education and promote lifelong learning opportunities for all	13 CLIMATE ACTION	Take urgent action to combat climate change and its impacts
5 GENDER EQUALITY	Achieve gender equality and empower all women and girls	14 LIFE BELOW WATER	Conserve and sustainable use of the oceans, seas and marine resources for sustainable development
6 CLEAN WATER AND SANITATION	Ensure availability and sustainable management of water and sanitation for all	15 LIFE ON LAND	Protect, restore and promote sustainable use of terrestrial ecosystems, sustainably manage forests, combat desertification, and halt and reverse land degradation and hart biodiversity loss
7 AFFORDABLE AND CLEAN ENERGY	Ensure access to affordable, reliable, sustainable and modern energy for all	16 PEACE, JUSTICE AND STRONG INSTITUTIONS	Promote peaceful and inclusive societies for sustainable development, provide access to justice for all and build effective, accountable and inclusive institutions at all levels
8 DECENT WORK AND ECONOMIC GROWTH	Promote sustained, inclusive and sustainable economic growth, full and productive employment and decent work for all	17 PARTNERSHIPS FOR THE GOALS	Strengthen the means of implementation and revitalize the global partnership for sustainable development
9 INDUSTRY, INNOVATION AND INFRASTRUCTURE	Build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation		

Figure 20. Sustainable Development Goals

Adapted from: (1)

Annexe II – Goal 2 - Zero Hunger: targets and indicators

ANNEXES

Annexe II – Goal 2 - Zero Hunger: targets and indicators

Table 17. Goal 2. End hunger, achieve food security and improved nutrition and promote sustainable agriculture

Targets		*Indicators*	
2.1	By 2030, end hunger and ensure access by all people, in particular the poor and people in vulnerable situations, including infants, to safe, nutritious and sufficient food all year round	2.1.1	Prevalence of undernourishment
		2.1.2	Prevalence of moderate or severe food insecurity in the population, based on the Food Insecurity Experience Scale (FIES)
2.2	By 2030, end all forms of malnutrition, including achieving, by 2025, the internationally agreed targets on stunting [12] and wasting [13] in children under-5 years of age (WHA65.6), and address the nutritional needs of adolescent girls, pregnant and lactating women and older persons	2.2.1	Prevalence of stunting (height for age <-2 standard deviation from the median of the World Health Organization (WHO) Child Growth Standards) among children under-5 years of age
		2.2.2	Prevalence of malnutrition (weight for height Z-score >+2 or Z-score <-2) among children under-5 years of age, by type (wasting and overweight)
2.3	By 2030, double the agricultural productivity and incomes of small-scale food producers, in particular women, indigenous peoples, family farmers, pastoralists and fishers, including through secure and equal access to land, other productive resources and inputs, knowledge, financial services, markets and opportunities for value addition and non-farm employment	2.3.1	Volume of production per labour unit by classes of farming/pastoral/forestry enterprise size
		2.3.2	Average income of small-scale food producers, by sex and indigenous status
2.4	By 2030, ensure sustainable food production systems and implement resilient agricultural practices that increase productivity and production, that help maintain ecosystems, that strengthen capacity for adaptation to climate change, extreme weather, drought, flooding and other disasters and that progressively improve land and soil quality	2.4.1	Proportion of agricultural area under productive and sustainable agriculture
2.5	By 2020, maintain the genetic diversity of seeds, cultivated plants and farmed	2.5.1	Number of plant and animal genetic resources for food and agriculture

[12] A 40% reduction in the number of children under-five years of age who are stunted.

[13] Reduce and maintain childhood wasting to less than 5%

Targets		*Indicators*	
	and domesticated animals and their related wild species, including through soundly managed and diversified seed and plant banks at the national, regional and international levels, and promote access to and fair and equitable sharing of benefits arising from the utilization of genetic resources and associated traditional knowledge, as internationally agreed		secured in either medium or long-term conservation facilities
		2.5.2	Proportion of local breeds classified as being at risk, not-at-risk or at unknown level of risk of extinction
2.A	Increase investment, including through enhanced international cooperation, in rural infrastructure, agricultural research and extension services, technology development and plant and livestock gene banks in order to enhance agricultural productive capacity in developing countries, in particular least developed countries	2.A.1	The agriculture orientation index for government expenditures
		2.A.2	Total official flows (official development assistance plus other official flows) to the agriculture sector
2.B	Correct and prevent trade restrictions and distortions in world agricultural markets, including through the parallel elimination of all forms of agricultural export subsidies and all export measures with equivalent effect, in accordance with the mandate of the Doha Development Round	2.B.1	Producer Support Estimate
		2.B.2	Agricultural export subsidies
2.C	Adopt measures to ensure the proper functioning of food commodity markets and their derivatives and facilitate timely access to market information, including on food reserves, in order to help limit extreme food price volatility	2.C.1	Indicator of food price anomalies

Annexe III – Assessing weight and length/height in children

Annexe III – Assessing weight and length/height in children

The basic anthropometry measurements include weight, length or height (depending on the child´s age).

Weight

Children should be weighed naked (especially babies) or, when socially inacceptable, with as less clothing as possible. If infants, if a diaper is worn, its weight is subtracted from the observed weight. It is recommended to use an electronic scale with a precision of 0.1kg (100g).

If the child is less than two years old or is unable to stand, it is better to use a scale that allows tared weighing (reset to zero with a person still on the scale). Thus, while remaining on the scale, the person holds the child and the scale gives the child´s weight (Figure 21-A). If the child is two years of age or more, the child can be weighed alone if she or he is capable of standing (Figure 21-B)

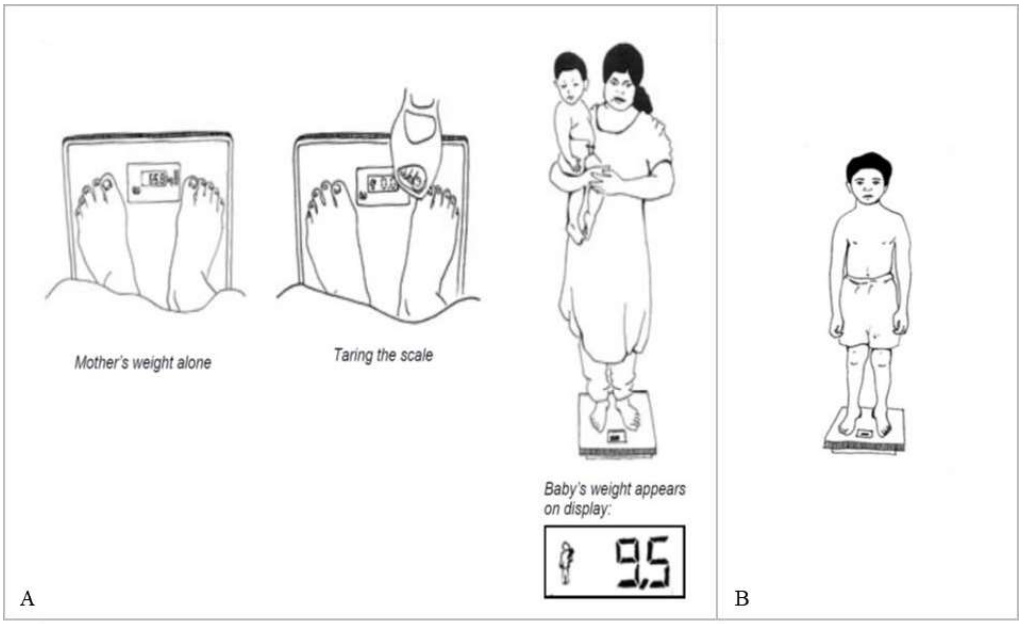

Figure 21. Weighing a child

(A) – Weighing a child under two years of age (or incapable to stand); (B) – Weighing a child with two or more years old. Adapted from (2)

Length and height

Ideally, length/height measurements should be assessed after weighing because the child is already with the clothes off, especially shoes, socks and hair ornaments. In children under two years of age the length is measured using a infantometer in the recumbent position (lying down), Figure 22. In older children (two or more years), the height is measured standing using a stadiometer, Figure 23.

In both cases, technicians must ensure that children do not have shoes, socks or hair ornaments during measurements, and the head should be positioned according to the Frankfurt position so that an imaginary vertical line from the ear canal to the lower border of the eye socket is perpendicular to the board. In general, standing height is about 0.7 cm less than recumbent length (3). Whether measuring length or height, caregiver collaboration is important to keep the child calm and in the right position.

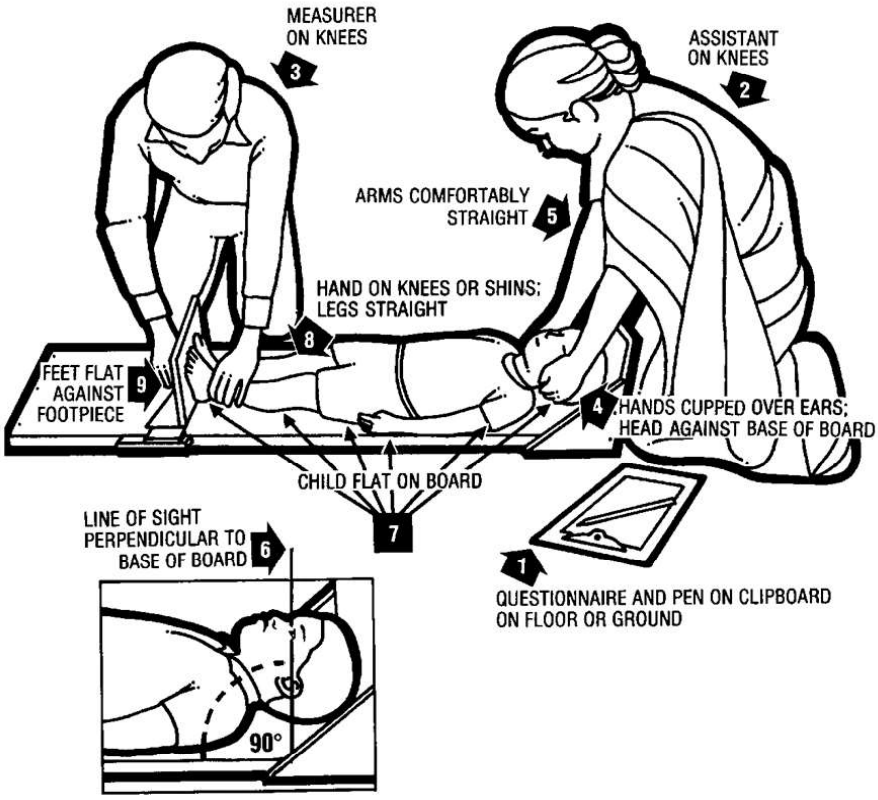

Figure 22. Measuring length of children less than two years

Source: (4)

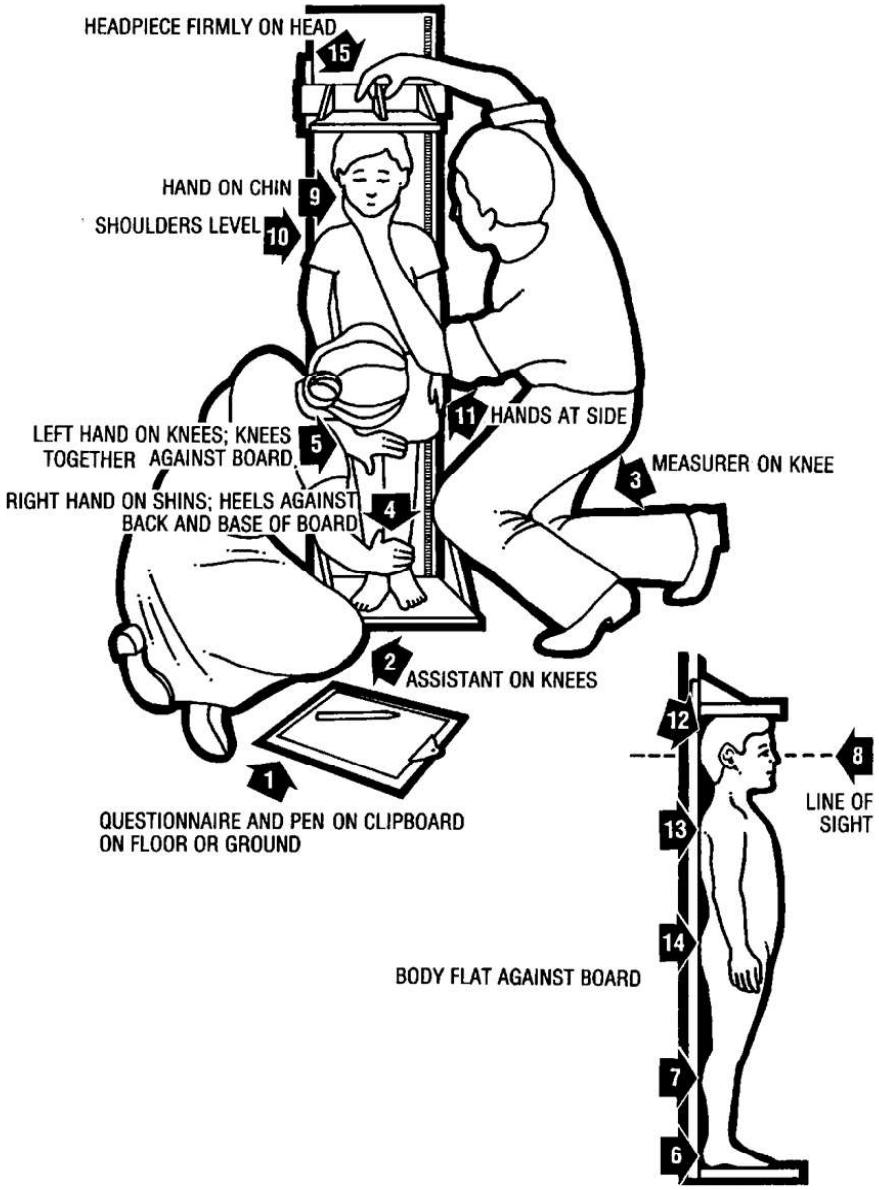

Figure 23. Measuring height of children with two years or more.

Source: (4)

Annexe IV – Summary of the thesis results

Annexe IV – Summary of the thesis results

The main findings of this thesis are presented in Table 18.

Table 18. Main findings of the thesis

Paper	Title	Main findings
I	**Etiology of diarrhoea in children younger than 5 years attending the Bengo General Hospital in Angola**	**Sociodemographic characterization** • 344 children included between September 2012 and December 2013 • mean age of children 15.5 months ± 12.4 SD • River (27.0%) was the most frequent drinking water source • No latrine existence in 20.3% of household **Malnutrition** • Wasting: mild (19.5%), moderate (11.1%), and severe (20.4%) • Stunting: mild (22.2%), moderate (15.9%), and severe (16.5%) • Underweight: mild (26.6%), moderate (19.5%), and severe (15.0%) **Diagnosis of enteric infections** • An enteric pathogenic agent was identified in 66.6% of samples • *Cryptosporidium* spp. (30.0%), rotavirus (25.1%), *Giardia lamblia* (21.6%), diarrheagenic *Escherichia coli* (6.3%), *Ascaris lumbricoides* (4.1%), adenovírus (3.8%), *Strongyloides stercoralis* (3.5%), astrovirus (2.6%), *Hymenolepis nana* (1.7%), *Entamoeba histolytica/díspar* (0.9%), *Taenia* spp. (0.6%), *Trichuris trichiura* (0.3%), and *Entamoeba histolytica* (0.3%) **Virus:** • Rotavirus the most frequent viral agent. • Rotavirus independently associated with age (<12 months, OR:5.0, 95% CI 2.7-9.3), type of admission (outpatients, OR:0.5, 95% CI: 2.7-9.3) and vomiting (yes, OR: 2.7, 95% CI: 1.5-4.8). Association with wasting only in the univariate analysis. **Parasites:** • Intestinal protozoa were more frequent than helminths • *Cryptosporidium* spp. independently associated with age (<12 months, OR: 3.5) • Helminths more common in older children (12-59 months, p=0.02)

Paper	Title	Main findings
		• *Strongyloides stercoralis* independently associated with maternal education (with education, OR: 0.2, 95% CI: 0.1-0.7) • All 6 children infected with *Hymenolepis nana* were stunted • No significant differences were found between protozoa and malnutrition **Bacteria:** • Suspicious colonies of *Escherichia coli* were isolated in 140 and 19 were confirmed to be pathogenic: EAEC (4.0%) and ETEC (2.3%) • *Shigella* spp., *Salmonella* spp., and *Campylobacter jejuni* were not isolated **Water and sanitation** • No significant association was found between any of the pathogenic agents and water sources, water treatment or latrine usage.
II	**Characterization of rotavirus infection in children with acute gastroenteritis in Bengo province, Nortwestern Angola, prior to vaccine introduction**	**Characterization** • Between September 2012 and December 2013, 342 stool samples were tested for rotavirus by immunochromatographic rapid test, and 86 (25.1%) were positive • Of the total positive samples, 72 (83.7%) were genotyped <u>Genotypes</u>: • G1P[8] 47.2% • G1P[6] 29.2% • G2P[4] 2.5% • Two G-types were found: G1 (83.3%) and G2 (15.3%). It was not possible to determine G-type for one sample. • Three P-types: P[8]: 47.2%; P[6]: 30.6%; and P[4]:12.5%. It was not possible to determine P-type for seven samples. • SGI: 15.3%, and SGII: 84.7% <u>Risk factors</u>: • The mean age of children was significantly lower for children with rotavirus compared to those not infected (9.2±5.00 vs 17.6±13.40 months)

Paper	Title	Main findings
		• Rotavirus infections was significantly more frequent in children who were being breastfed (exclusive or continued) compared to those who had already been weaned or had never been breastfed (34.2% vs 3.9%) • No association was found between rotavirus infection and maternal education, drinking water source and treatment method applied, sanitation facilities, underweight, fever, signs of dehydration, hospitalization, duration of diarrhoea, and seasonality.
III	**Molecular characterization of *Giardia lamblia* in children less than 5 years of age with diarrhoea attending the Bengo General Hospital, Angola.**	**Characterization** • Between September 2012 and December 2013, the diagnosis of *Giardia lamblia* was performed in 344 stool samples, through microcopy and antigen rapid test, and 73 (21.6%) were positive for at least one of the methods. **Molecular charaterization** • DNA extraction was performed in 63 (86.3%) • 16 were amplified for the ssu-rRna, and 11 successfully sequenced • Samples belonging to assemblage B (11/12) – 91.7% • Samples belonging to assemblage A (1/12) – 8.3% • It was not possible to determine the subassemblages belonging to assemblage B due to high polymorphism observed • Isolate belonging to assemblage A was found to belong to the A3 subassemblage
IV	**Two-year impact of annual albendazole versus four-monthly test-and-treat approach of intestinal parasites on children nutritional**	**Characterization** • Between December 2013 and December 2014, 692 children were recruited, and 121 children were included and randomly assigned to: o Arm 1 (A1) – 29 (ALB, individual level) o Arm 2 (A2) – 31 (ALB, household level) o Arm 3 (A3) – 31 (diagnosis + treatment, individual level) o Arm 4 (A4) – 30 (diagnosis + treatment, household level) **Baseline** • sociodemographic, anthropometric and clinical characteristics were similar across arms. Of the total (N=121): o 95.0% were from urban areas o 29.1% with unimproved drinking water source

Paper	Title	Main findings
	status in Bengo, Angola: a four-arm randomised parallel trial	○ 19.7% without a latrine ○ Mean member per household 5.97 ± 2.08 SD ○ 74.4% with less than 3 rooms per household ○ 74.4% living in houses made of adobe <u>Malnutrition</u> ○ Stunting: mild (33.1%), moderate (20.7%), and severe (9.9%) ○ Wasting: mild (21.5%), moderate (5.0%), and severe (2.5%) ○ Underweight: mild (29.4%), moderate (12.6%), and severe (3.4%) ○ Mean HAZ (-1.34±1.33) ○ Mean WHZ (-0.29±1.18) ○ Mean WAZ (-0.80±0.98) ○ More stunting in children living in houses with adobe walls compared with those made of bricks (39.1% vs 10.0%, p=0.003, OR=4.2). ○ More stunting in children living in houses with earth or sand floors than those with ceramic floors (60% vs 27.5%, p=0.017; OR=1.2). <u>Intestinal parasites</u> ○ *Giardia lamblia* (57.0%), *Ascaris lumbricoides* (25.6%), *Strongyloides stercoralis* (13.2), *Trichuris trichiura* (5.8%), *Hymenolepis nana* (5.8%), *Cryptosporidium* spp. (5.8%), *Entamoeba histolytica* (2.5%) ○ diarrhoea (47.9%), fever (80.2%), vomiting (14.9%); ○ mean haemoglobin: 10.6 ± 1.67 g/Dl ○ Malaria (*P.falciparum* – rapid test) **Follow-up** • 12 permanently lost to follow-up • Temporary withdrawal occurred in all arms **Follow-up: Interventions - Arm 1** • 28 (96.6%) children received a single dose of ALB at Fu1; • 24 (85.7%) children received a single dose of ALB at Fu4 • 24 (82.8%) received complete intervention in both Fu1 and Fu4

Paper	Title	Main findings
		Follow-up: Interventions - Arm 2 • 28 (90.3%) children and 126 household members received ALB in Fu1 • 27 (87.1%) children and 152 household members received the allocated treatment in Fu4 • In sum, the intervention was completely received by 22 children (71%) in both time points, with a household coverage ⩾80%. **Follow-up: Interventions - Arm 3** • the number of those delivering a stool sample and receiving appropriate treatment was 24 (77.4%) in Fu1, 21 (67.7%) in Fu2, 23 (74.2%) in both Fu3 and Fu4, 20 (64.5%) in Fu5, and 22 (71.0%) in Fu6. • Only 15 (48.4%) children completed intervention by delivering a stool sample in all follow-ups and receiving appropriate treatment. **Follow-up: Interventions - Arm 4** • the number of children delivering a stool sample and receiving treatment was 22 (73.3%) in Fu1, 24 (80.0%) in Fu2, 25 (83.3%) in Fu3, 23 (76.7%) in Fu4, 25 (83.3%) in Fu5, and 22 (73.3%) in Fu6. • The percentage of household members delivering the requested stool sample during the follow-up period ranged between 63.0% (92/146) and 74.6% (94/126). • At the household level, only nine children (30%) and at least 50% of their household members were able to deliver a stool sample and receive appropriate treatment in the six follow-ups. **Non-parametric approach for longitudinal data** (*npar*LD): • no significant effects were obtained in any of the interventions (effect of arm), nor by arm*time interaction, table 3. However, temporal changes occurred in all nutritional outcomes ($p<0.05$) **Modeling for longitudinal data**: • The results from GEE and LMM models were very similar, even for different correlation structures, enhancing significant temporal changes in primary outcomes

Paper	Title	Main findings
		For secondary outcomes: • no significant differences in the rates of infection between A3 and A4 • *Giardia lamblia* was the most frequent parasite identified in each time • From Fu1 to Fu6, there was a significant reduction of parasitic infection in A4 (p=0.039), but not A3 (p=0.727). • At the end, the reduction of *Giardia lamblia* infection was higher in A4 (from 37.5% to 16.7%, p=0.07) than A3 (from 34.6% to 30.4%, p=0.727). • After requiring a stool sample to all children in the last Fu, infections rates for *G. lamblia* were lower in A4 (16.7%), followed by A3 (30.4%), A1 (34.8%), and A2 (40%); but for *A. lumbricoides* they remained similar in all arms (near 9% for A1 and A2, 10% in A3, and 8% in A4).

Annexe V – Fieldwork photos

Annexe V – Fieldwork photos

Figure 24. Outpatient health unit of Hospital Geral do Bengo, Bengo province, Angola

Figure 25. Hospital Municipal do Dande, Bengo province, Angola

Figure 26. Posto Médico o Bom Samaritano, província do Bengo, Angola

Figure 27. Data collection

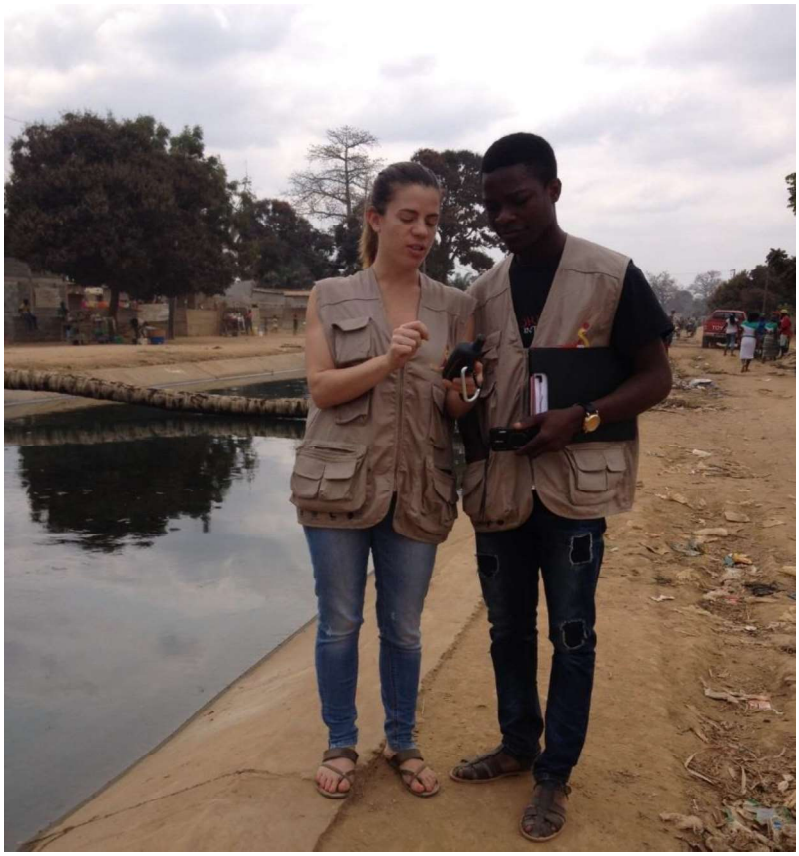

Figure 28. Using the global positioning system (GPS) for houses location

Figure 29. On the way for a community follow-up assessment

Figure 30. On the way for a community follow-up assessment

Figure 31. Entering a house for community follow-up assessment

Figure 32. Community follow-up assessment

Figure 33. Assessing haemoglobin concentration

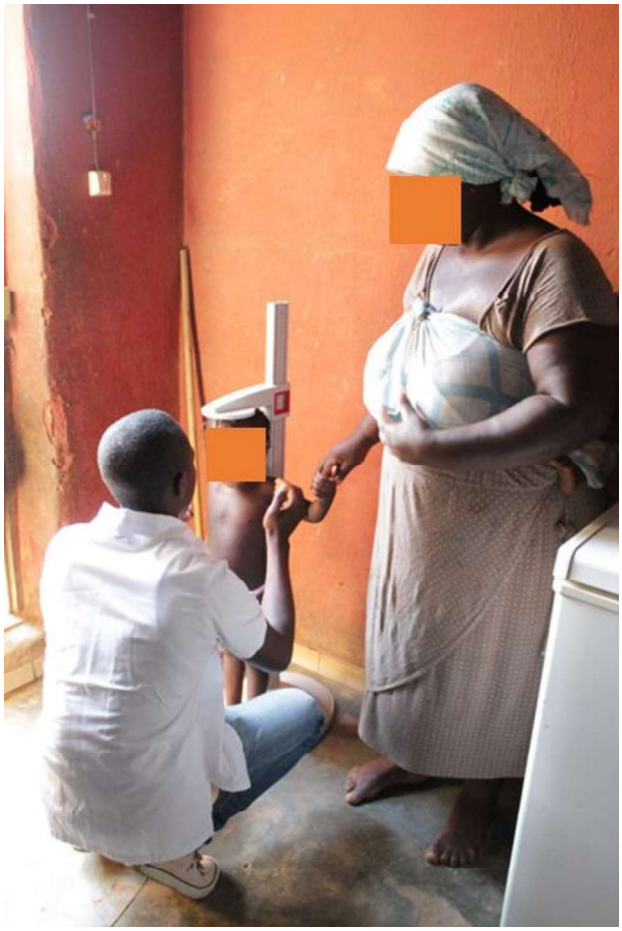

Figure 34. Assessing a child´s height during a community follow-up assessment

Figure 35. Difficulties in accessing houses during community follow-up

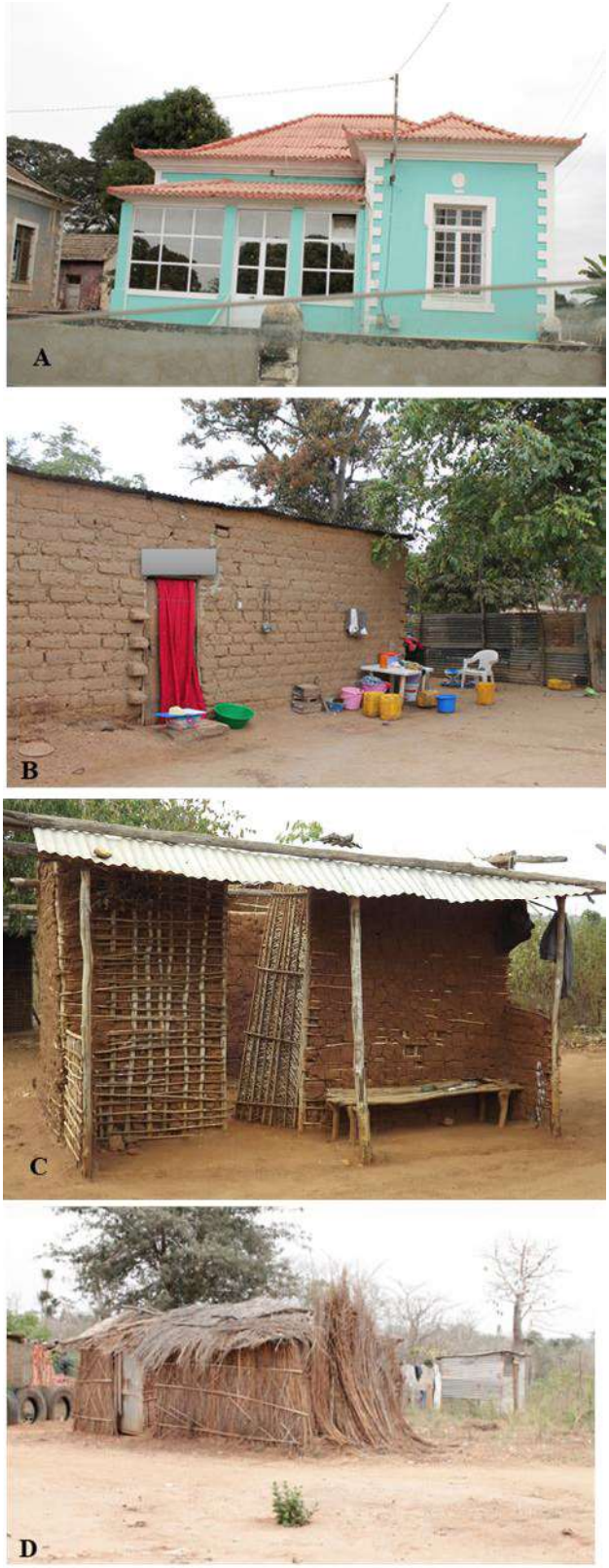

Figure 36. Household construction materials in the Dande HDSS area

A - Block walls and roof tile; **B**- Adobe walls and iron sheet roof; **C-** Wattle and daub walls and iron sheet roof; **D** - Straw walls and roof

Figure 37. Common water sources for drinking, and/or bathing, and/or other activities

A-Irrigation channel; **B**- Public tap water; **C**- river; **D** - tank

Figure 38. Laboratory at the Health Research Centre of Angola (CISA), Bengo, Angola

Figure 39. CISA´s multidisciplinary field team

Annexes references

1.United Nations (UN) General Assembly. Transforming our world: the 2030 Agenda for Sustainable Development. A/RES/70/1. United Nations: 21 October 2015 [Accessed 20 June 2018]. Available from: https://sustainabledevelopment.un.org/index.php?page=view&type=111&nr=8496&menu=35].

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