Cláudia Patrícia Fançony Videira

Effectiveness of Nutrition and WASH/malaria educational community-based interventions in reducing anemia, malnutrition and micronutrient deficiencies in preschool children from Bengo, Angola.

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FMUP FACULDADE DE MEDICINA
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Ao abrigo do Art.º 4º do Decreto-Lei n.º 707/2018, fazem parte desta dissertação as seguintes publicações e manuscritos:

I. Fançony CL; Lavinha J.; Brito, M.; and Barros, H.: Anemia in preschool children from Angola: a review of the evidence. *Porto Biomedical Journal* 2020, 5:1(e60):9.2).
II. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Iron deficiency anemia among 6-to-36-month children from northern Angola. *BMC Pediatr* 2020, 20:298.
III. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Zinc deficiency interacts with intestinal/urogenital parasites to cause anemia in children from Bengo – Angola. (In elaboration).
IV. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Efficacy of Nutrition and WASH/Malaria Educational Community-Based Interventions in Reducing Anemia in Preschool Children from Bengo, Angola: Study Protocol of a Randomized Controlled Trial. Int J Environ Res Public Health 2019, 16 (3).
V. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Effectiveness of nutrition and WASH/malaria educational community-based interventions in reducing anemia in children from Angola: a cluster-randomized controlled trial, (submitted to the Scientific Reports Journal).

Declaro que colaborei no desenho dos estudos e definição dos objetivos de todos os trabalhos que compõem a presente tese. Adicionalmente, participei activamente na recolha de dados, na análise estatística e interpretação dos resultados. Fui responsável por redigir as versões iniciais dos manuscritos, rever as versões subsequentes e elaborar as versões finais de todos os manuscritos aqui apresentados.

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O Júri da Prova foi nomeado pelo despacho FOA.26.1600.2020, de 2020.03.03, proferido no âmbito da delegação reitoral.

À Ana Kataleya Fançony Videira Calonga

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Table of contents

List of original publications

This doctoral dissertation is based in the 5 papers described bellow, which will be referred to by their Roman numerals. The first paper (paper I) have supported mainly the construction of the state of art and interpretations of results. Papers II and III corresponded to specific objectives, paper IV guided the methodological part of the investigation and paper V corresponded to the main research question being investigated.

I. Fançony CL; Lavinha J.; Brito, M.; and Barros, H.: Anemia in preschool children from Angola: a review of the evidence. *Porto Biomedical Journal* 2020, 5:1(e60):9.2).
II. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Iron deficiency anemia among 6-to-36-month children from northern Angola. *BMC Pediatr* 2020, 20:298.
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V. Fancony C, Soares A, Lavinha J, Barros H, Brito M: Effectiveness of nutrition and WASH/malaria educational community-based interventions in reducing anemia in children from Angola: a cluster-randomized controlled trial (submitted to Scientific Reports Journal).

Abstract

Introduction

There is an important lack of scientific evidences and systematized knowledge in the indexed literature, regarding the etiologic profile of anemia in Angola. Also, nutrition-specific, and nutrition-sensitive interventions targeting this condition are rare within the country. This knowledge is essential to inform and optimize national public health policies willing to address it.

In order to address these questions, we systematically reviewed the literature on Anemia in the country, summarized results published in Africa and designed a comprehensive cluster-randomized controlled trial to study the efficacy of educational-plus-therapeutic interventions in Nutrition and in Water, Sanitation and Hygiene (WASH)/Malaria in reducing anemia. This trial, comprehended both cross-sectional and longitudinal analysis of the frequency and anemia´s determinants, specifically aiming at 1) describing the dynamics of anemia in Angolan and African children population; 2) measuring the prevalence of anemia and its etiologies; 3) describing the main factors separately associated with the occurrence of Iron Deficiency Anemia (IDA) and Iron-unrelated Anemia (non-IDA), 4) investigating the impact of zinc deficiency and overload in the occurrence of anemia and, 5) determining the effectiveness of an educational intervention in Water, Sanitation, Hygiene and Malaria in reducing nutritional anemia, when compared with an educational intervention in Nutrition.

Methods

At the beginning of the study, pre-intervention data (on demography, socio-economy, parental practices associated with water, sanitation, hygiene, malaria and infant and young child feeding, and children´s anthropometric, nutritional, parasitological, biochemical and molecular) was collected from all children under 3 years old resident in hamlets with functional health posts, recruited through a census approach. At this evaluation moment, a test-and-treat approach was implemented aiming at clearing children from malaria, intestinal parasites, and schistosomiasis infections. Then, the hamlets (our cluster units) were randomized to each of the 3 study arms (control, WASH/Malaria, and nutrition) and the families from the educational intervention arms were provided with six personalized counselling sessions at their homes. Process indicators were collected during those visits to determine changes in the proportion of adequate behaviour/practices between the 1st and 6th educational visits at the Nutrition and the WASH/Malaria arms. Post-intervention waves, following the same structure as the pre-intervention assessment, were implemented both 6 and 12 months after the beginning of the study.

The initial assessment of participants occurred between March and May of 2015 (baseline that included 948 children), while the follow-ups occurred between November and December 2015 (6-month follow up that included 524 children) and between July and August of 2016 (12-month follow up that included 660 children). The counselling moments have intercalated those evaluation moments in 2 rounds of 3 counselling visits. The first occurred between June and October 2015 (included 610, 576 and 574 of the caretakers) and the second round occurred between January and June of 2016 (included 561, 574 and 563 of the caretakers).

Results

In summary, 44.4% of children (under-3 years of age) had hemoglobin bellow 11g/dl at the baseline (46.0% of which also had iron deficiency). Adjusted multivariate regression models associated IDA with age, gender and inflammation, while associating non-IDA with age, zinc deficiency and overload, *Plasmodium falciparum* and sickle cell trait/anemia.

In turn, children with high zinc levels were significantly less likely of being infected with *P. falciparum*, while children with zinc deficiency had 1.9 more chances of having non-malarial inflammation. Nevertheless, we observed: 1) no mediator or interactive effects among zinc deficiency and iron deficiency, 2) no significant evidences that inflammation could be mediating or interacting with zinc deficiency to cause anemia and 3) no mediator, but a significant interaction effect between zinc deficiency and infections (having at least one intestinal or urogenital parasite) in the causal pathway to anemia (OR: 13.26, p= 0.022 for the interaction between zinc deficiency and parasites, OR: 2.30, p= 0.007 for direct effect between anemia and Zinc Deficiency, and OR: 1.57, p= 0.182 for direct effect between parasites and anemia).

Facing operational limitations observed at the baseline, we moved from an efficacy to an effectiveness cluster-randomized controlled trial. Nevertheless, some design aspects from the original published protocol were sustained and implemented, such as standardized design, structured interventions, focus on specific primary outcomes and randomized assignment to interventions. In summary, while there were no significant differences in the longitudinal variations of anemia and hemoglobin, between both educational and control groups (in either crude or adjusted difference-in-difference models), the WASH/Malaria group was observed to have 22.8% higher prevalence of anemia than the nutrition group. However, had also higher prevalence of *P. falciparum.*

Additional ancillary analysis in each group, allowed specifying that despite that no relevant differences between groups were observed, there were increased hemoglobin for the control, nutrition and WASH/Malaria groups (an increase of 0.05 g/dl, 0.27 g/dl and 0.13 g/dl, respectively) and a decreased prevalence of anemia (5.50%, 14.2% and 5.4% lower prevalence) between the pre-and-post intervention moments. However, statistically significant only in the nutrition group. Those results are

in line with our process indicators, reporting that 70% of the participants successfully received 5-to-6 visits and that higher proportion of adequate behavior/practices were observed in both educational groups (WASH/Malaria and Nutrition groups), between the 1th and 6th domiciliary counselling visits.

Discussion and conclusion

The main factors associated with IDA and non-IDA within this geographic setting were already commonly reported in Africa, presenting however differences in their contribution between areas of the continent. Those differences were suggested to be related with the predominance of etiologic factors, specific of those settings. In age groups, our results suggest that the effect of both inadequate young feeding practices and prolonged nutritional restriction may have been exacerbated by high iron requirements originating from rapid growth and physiological state, having a major impact in the occurrence of IDA. On the other hand, possible inadequate practices or behaviours may have exacerbated the occurrence of infections and infection-related non-IDA, that may in turn have contributed to an apparent nutritional immunity associated with zinc levels.

Results from our models suggest that implementing two semesterly rounds of an exclusive test-and-treat therapeutic approach, have no statistically significant difference in reducing anemia and increasing hemoglobin, as compared to combining this approach with 6 monthly domiciliary counselling visits addressing adequate child nutrition or WASH/Malaria practices. Considering the positive results reported by others, and the fact that our process indicators suggest the occurrence of some degree of behavior change in both educational intervention groups, these effectiveness results could suggest either that, 1) the therapeutic component of the intervention played in fact the major role and the educational component had a residual effect, or 2) not all educational actions have been translated into changed behavior and in consequence the beneficial results happened incompletely and/or 3) the educational actions lacked the required intensity and duration of the educational actions (only 12-months) to translate into significant changes in the primary outcomes. These aspects should be considered when designing similar interventions.

Resumo

Introdução

Existe uma importante falta de evidências científicas e conhecimento sistematizado na literatura indexada, relativamente ao perfil etiológico da anemia em Angola. Além disso, intervenções direcionadas para essa condição, que por sua vez tenham em conta as causas directas ou indirectas de malnutrição são raras no país. No entanto, esse conhecimento é essencial para informar e optimizar as políticas nacionais de saúde pública que visem prevenir ou controlar a anemia.

Para abordar estas questões, fizemos uma revisão sistemática da literatura sobre anemia no país, resumimos os resultados publicados em África e desenhamos um ensaio clínico, controlado randomizado para estudar a eficácia das intervenções educacionais e terapêuticas, em Nutrição e em Água, Saneamento, Higiene e Malária (WASH/Malaria), na redução da anemia. Este estudo compreendeu análises transversais e longitudinais da frequência e determinantes da anemia, visando especificamente 1) descrever a dinâmica da anemia na população infantil angolana e africana; 2) medir a prevalência de anemia e suas etiologias; 3) descrever os principais factores associados separadamente à ocorrência de anemia ferropriva e anemia não relacionada a deficiência em ferro, 4) investigar o impacto da deficiência e excesso de zinco na ocorrência de anemia e, 5) determinar a efectividade de uma intervenção educacional em boas práticas de Água, Saneamento, Higiene e Malária na redução da anemia nutricional, quando comparada com uma intervenção educacional em Nutrição.

Métodos

No início do estudo, dados pré-intervenção (sobre demografia, socioeconómica, práticas parentais associadas à água, saneamento, higiene, malária e alimentação de crianças em idade pré-escolar, dados antropométricos, nutricionais, parasitológicos, bioquímicos e moleculares) foram recolhidos de todas as crianças menores de 3 anos residentes em aldeias com postos de saúde funcionais, recrutadas por uma abordagem do tipo censos. Nesse momento da avaliação, foi implementada uma abordagem *test-and-treat* com o objetivo de tratar infecções por malária, parasitas intestinais e schistosomíase. Em seguida, as aldeias (unidades de cluster) foram randomizadas para cada um dos três braços de estudo (controlo, WASH/Malária e Nutrição) e as famílias dos braços de intervenção educacional receberam seis sessões de aconselhamento personalizado em suas casas. Foram recolhidos indicadores de processo durante estas visitas para determinar mudanças na proporção de comportamentos/práticas adequadas entre a 1ª e a 6ª visitas educacionais nos braços de Nutrição e WASH/Malária. Os *follow ups* pós-intervenção, seguiram a mesma estrutura da pré-intervenção, e foram implementadas 6 e 12 meses após o início do estudo.

A avaliação inicial dos participantes ocorreu entre março e maio de 2015 (*baseline* que incluiu 948 crianças), enquanto que o *follow up* 6 meses ocorreu entre novembro e dezembro de 2015 (e incluiu 524 crianças) e o *follow up* 12 meses ocorreu entre julho e agosto de 2016 (incluindo 660 crianças). Os momentos de aconselhamento domiciliar intercalaram os momentos de avaliação em 2 rondas de 3 visitas. A primeira ronda ocorreu entre junho e outubro de 2015 (incluiu 610, 576 e 574 dos cuidadores) e a segunda ronda ocorreu entre janeiro e junho de 2016 (incluiu 561, 574 e 563 dos cuidadores).

Resultados

Em resumo, 44.4% das crianças (menores de 3 anos) tinham hemoglobina abaixo de 11g/dl na *baseline* (46.0% das quais também tinham deficiência de ferro). Modelos de regressão multivariada ajustados associaram a anemia ferropriva com idade, sexo e inflamação, enquanto associaram a anemia não relacionada com o ferro (não ferropriva) com a idade, deficiência e excesso de zinco, *Plasmodium falciparum* e traço/anemia falciforme.

Por sua vez, crianças com altos níveis de zinco apresentaram menor probabilidade de estarem infectadas com *P. falciparum*, enquanto que crianças com deficiência de zinco tiveram 1,9 vezes mais chances de apresentar inflamação não-malárica. Adicionalmente, não observamos: 1) nenhum efeito mediador ou interativo entre a deficiência em zinco e a deficiência em ferro, 2) nenhuma evidência significativa de que a inflamação poderia estar a mediar ou interagir com a deficiência em zinco para causar anemia e 3) nenhum efeito mediador entre a deficiência em zinco e a ocorrência de infecções (pelo menos um parasita intestinal ou urogenital). No entanto, 4) foi observada interação significativa entre a deficiência em zinco e a ocorrência de infecções, aumentando significativamente o risco de anemia (OR: 13.26, p = 0.022 para a interação entre deficiência de zinco e parasitas, OR: 2,30, p = 0.007 para efeito direto entre anemia e deficiência em zinco e OR: 1.57, p = 0.182 para o efeito direto entre parasitas e anemia).

Diante das limitações operacionais observadas na *baseline*, passamos de um estudo de eficácia para um ensaio clínico randomizado controlado de efectividade. No entanto, alguns aspectos do protocolo original publicado foram mantidos e implementados, como por exemplo a padronização do projecto, intervenções estruturadas, foco em resultados primários específicos e atribuição aleatória de intervenções. Em resumo, embora não houvesse diferenças significativas nas variações longitudinais de anemia e hemoglobina, entre os grupos educacional e controlo (em nenhum dos modelos bruto ou ajustado), observou-se que o grupo WASH/Malária teve 22,8% maior prevalência de anemia do que o grupo de Nutrição. No entanto, também houve maior prevalência de *P. falciparum nesse grupo*.

Análises auxiliares adicionais em cada grupo permitiram especificar que, apesar de não serem observadas diferenças relevantes entre os grupos, houve aumento da hemoglobina nos 3 grupos (aumento de 0.05 g/dl, 0.27 g/dl e 0,13 g/dl, respectivamente para o Controlo, Nutrição e

WASH/Malária) e diminuição da prevalência de anemia (de 5.50%, 14.2% e 5.4%, respectivamente) entre os momentos pré e pós-intervenção. No entanto, a significância estatística observou-se apenas no grupo de nutrição. Estes resultados alinham-se com os indicadores de processo, que relatam que 70% dos participantes receberam com êxito 5 a 6 visitas (num total de 6) e que uma proporção maior de comportamentos/práticas adequadas foi observada nos dois grupos educacionais (grupos WASH / Malária e Nutrição), no final do estudo.

Discussão e conclusão

Os principais factores associados com a anemia ferropriva e com a anemia não ferropriva que reportamos aqui já tinham sido relatados em África, apresentando, no entanto, diferenças na sua contribuição entre as áreas do continente. Sugere-se que estas diferenças estejam relacionadas com a predominância de factores etiológicos específicos desses contextos. Nos grupos etários, os nossos resultados sugerem que o efeito de práticas inadequadas de alimentação e restrição nutricional prolongada pode ter exacerbado as elevadas necessidades de ferro originados pelo rápido crescimento e estado fisiológico. Este facto pode ter tido um impacto considerável na ocorrência de anemia ferropriva. Por outro lado, as possíveis práticas ou comportamentos inadequados de WASH/Malaria podem ter exacerbado a ocorrência de infecções, contribuindo a ocorrência e anemia não ferropriva e uma aparente imunidade nutricional associada aos níveis de zinco.

Os resultados dos nossos modelos sugerem que a implementação de duas rondas semestrais de desparasitação não apresenta diferença estatisticamente significante na redução da anemia e no aumento da hemoglobina, em comparação com a combinação dessa abordagem com 6 visitas de aconselhamento domiciliar mensal, tanto abordando a Nutrição infantil como as práticas de WASH/Malária. Considerando os resultados positivos relatados por outros e o facto de que os nosso indicadores de processo sugerem a ocorrência de algum grau de mudança de comportamento nos dois grupos de intervenção educacional, esses resultados de efetividade podem sugerir que: 1) a componente terapêutica da intervenção desempenha de facto o papel principal, sendo que a componente educacional tive apenas um efeito residual, ou 2) nem todas as acções educacionais foram traduzidas em mudança de comportamento e, consequentemente, os resultados benéficos ocorreram incompletamente e/ou 3) as acções educacionais careciam da intensidade e duração necessárias das acções educativas para se traduzir em mudanças significativas nos resultados primários. Esses aspectos devem ser considerados ao desenhar e planear intervenções semelhantes.

Abbreviations

ALB Albendazole
BMI - Body Mass Index
CISA - Health Research Center of Angola (translated)
CRP - C-reactive protein
FPA - Food Photography Atlas
G6PD Glucose Phosphate Dehydrogenase
HAZ - Height-for-age
Hb – Hemoglobin
HDSS - Health, Demographic and Surveillance System
HPLC - High-performance Liquid Chromatography
ID - Iron Deficiency
IDA - Iron Deficiency Anemia
IYCF - Infant and Young Child Feeding
MDD - Minimum Dietary Diversity
Non-IDA – Non-Iron Deficiency Anemia
NTD – Neglected Tropical Diseases
PCR – Polymerase Chain Reaction
PZQ - Praziquantel
RCT - Randomized Controlled Trial
STH Soil-Transmitted Helminth
UNICEF - United Nations Children's Fund
WASH - Water, Sanitation and hygiene
WAZ - Weight-for-age
WHO World Health Organization
WHZ - Weight-for-height

Anemia is a condition that expresses pathologic processes, that is characterized by decreased number of circulating red blood cells, often accompanied by a reduction on its hemoglobin concentration or altered morphology leading to a reduction of blood oxygen-carrying ability [1-4]. Chronic anemia in children is associated with limitations in the cognitive, physical (impaired physical coordination) and psychomotor development, and physical growth, increased susceptibility to infection, subsequent implications in health and social aspects of adult life (such as loss of productivity from impaired work capacity, and consequent economic loss) [1, 2, 4-7].

Prevalence of anemia in Africa, Angola and Bengo

The prevalence of anemia for 6-to-59-month children from the World Health Organization´s (WHO) African region in 2011 was 62%, presenting a substantial geographic variability [8]. For instance, in that year, Tunisia, Libya, Algeria, Morocco, Seychelles and Rwanda had the lowest prevalence's in Africa (29-38%), followed by South Africa, Botswana, Djibouti, Mauritius, Swaziland, Egypt, Kenya, Burundi, Lesotho, Namibia, Ethiopia and Madagascar (41-50%), Comoros, Angola, Uganda, Somalia, Zambia, Eritrea, Sao Tome and Principe, Sudan, Zimbabwe, Cabo Verde and Gabon (51-60%), Tanzania, Cameron, Benin, Congo, Gambia, Malawi, Mozambique and Democratic Republic of Congo (61-67%) and the remaining countries of the continent having the highest prevalence's (71-86%) [8]. Temporal variations were also observed among those countries, presenting gender and age-specific variabilities [9-12]. For instance, the number of countries with prevalence´s higher than 50% decreased significantly between 1990 and 2010, decreasing more for males and having negative trends in under 5 years old children [2, 3, 13, 14].

In Angola, the southern African country with the highest prevalence rate in 1990, Kassebaum *et al.* 2016 reported a similar tendency of prevalence rate decreasing (from 50-60% to 40-50% for all ages) [13]. Prevalence between 1990 and 2010 for Angolan neonates was reported to be lower than both infants and 1-4 years children [3]. It was reported that in 1992 there was a high proportion (near 39%) of children aging between 3 months and 13 years with hemoglobin below 6.8mmol/l, occurring mainly in children from low social and economic state (in which the anemia frequency was in turn associated with physical development) [15]. Later, reports from the WHO, mention that the prevalence for Angolan preschool children between 1998 and 1999 was 29.7%, representative of a moderate public health problem [16]. Between 2015 and 2016, the national multiple indicators survey, reported alarming prevalences in under 5 years old children (65%) [17]. In that age group, the prevalence was reported to be higher in 6-to-11 months children (83% in 6-8 months and 82% in 9-11 months children) and to have higher severity in 12-17 months children with 9% of moderate-to-severe anemia [17, 18].

Additionally, geographic heterogeneity was documented, with the prevalence ranging from 50% in Lunda Sul and 77% in Cuando Cubango [18].

Regionally, representative studies conducted within the Dande municipality of the Bengo´s province in 2010 reported that the prevalence of anemia in under 5 years old children was 57% [19, 20]. Between 2012-2013, Lemos *et al* tested a therapeutic intervention in children (with 2-15 years) from a highly *Schistosoma haematobium* endemic setting of that province in which the prevalence of anemia at the baseline was very high (76%) [21].

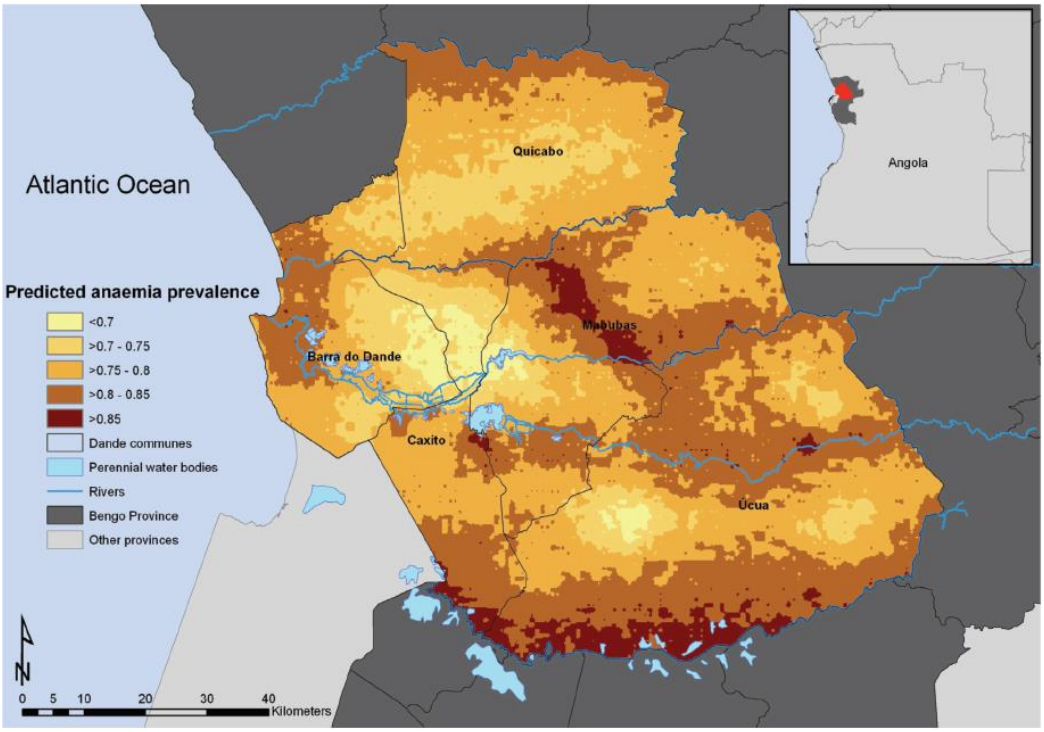

Figure 1 – Predicted spatial distribution of anemia for younger boys in Dande municipality, Angola. (Source: Soares Magalhães et al. 2013) [22].

Factors associated with anemia in Africa and Angola

The context-specific etiologic profile of anemia was suggested to be a crucial factor, influencing either the distribution of anemia and anemia´s associated morbidity and mortality [5, 23-27]. Cause-specific reports describe that despite being the greatest cause of anemia, the contribution of Iron Deficiency Anemia (IDA) for the total of anemia cases decreased from 66.2% in 1990 to 62.6% in 2013 worldwide [13]. In general, anemia in Africa is mainly caused by iron deficiency, followed by infections (mainly malaria, hookworm and schistosomiasis) and genetic causes (mainly pathogenic gene mutations leading to sickle cell , thalassemia and Glucose-6-phosphate dehydrogenase (G6PD) deficiency) [3, 13]. Furthermore, the contribution of each associated factor varies, and different etiologic profiles are

expected to occur within the continent. For instance, in the south sub-Saharan Africa, the contribution of hookworm is higher than in other African areas, while malaria and sickle cell are more relevant in the central and west sub-Saharan Africa (where malaria and the hemoglobinopathies were reported to collectively explain 80% of anemia cases), and schistosomiasis have higher contribution to anemia in the east [3, 13]. Among children aging between 28 days to 4 year, anemia was reported to be mainly caused by iron deficiency, infections (mainly malaria, hookworm and schistosomiasis) and genetic causes (mainly sickle cell anemia, thalassemia and G6PD deficiency) [3]. Older children within this age group, have higher contribution of infections (mainly hookworms) and lower contribution of iron deficiency [3].

Nationally, a strong relationship between anemia and malaria was evidenced by the Malaria Indicators Survey conducted in the country in 2011, suggesting that malaria-infected children were at a higher risk of anemia, and that areas with the highest prevalence of malaria had higher proportion of severe anemia [28]. Regionally, studies conducted within the Dande municipality of the Bengo´s province in 2010 reported that malaria and schistosomiasis were responsible for 16% and 10% of anemia cases, while ascariasis was associated with undernutrition (in turn responsible for 13% of anemia cases) [19, 20]. In that area, the distribution of anemia was found to be heterogeneous and to be influenced by the distribution of malaria. Genetically, results from a prospective newborn screening program for sickle cell anemia, described a prevalence of 21% of sickle cell trait and 1.5% with sickle cell anemia, while the determination of G202A, A376G and C563T mutations of the Glucose-6-phosphate dehydrogenase (G6PD) gene showed a considerable prevalence of disease-causing genotypes (10.9% of A-/ A- in girls and 20.6% of A- in boys) [29-31].

Rationale behind the 3 main causal pathways

Anemia is in general reported to be caused directly by 1) nutritional deficiencies, 2) infectious diseases and 3) genetic hemoglobin and other red blood cell disorders [32].

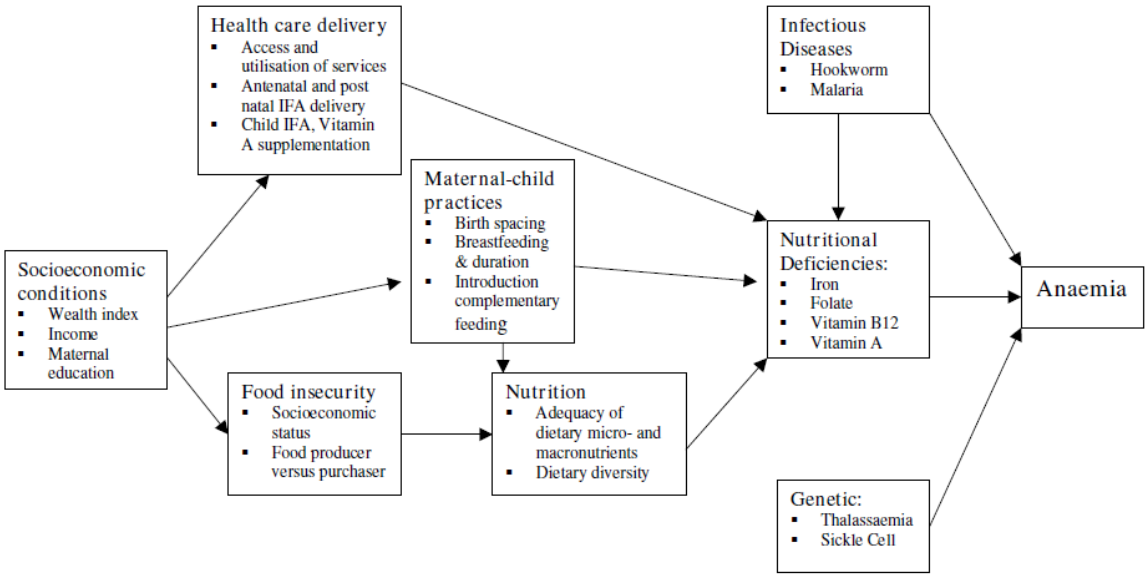

Figure 2 – Diagram with possible associations with anemia (Source: Pasricha *et al*. 2009)[32].

Anemia and nutritional deficiencies

While inadequate dietary intake is a primary pathway leading to anemia, nutritional anemias can also result from increased nutrient losses, impaired absorption and/or altered nutrient metabolism [4]. Iron deficiency is the most common cause of anemia and is estimated to contribute to approximately 42% of all cases among children under 5 years of age worldwide [4]. Furthermore, deficiencies of vitamins A, B2 (riboflavin), B6 (pyridoxine), B12 (cobalamin), C, D and E and folate can also result in anemia, due to their specific roles in production of hemoglobin or erythrocytes [4]. Additionally, other minerals such as zinc, selenium, copper, cobalt, molybdenum and magnesium as well as amino acids should be considered [33]. For instance, there are some divalent metals that share iron´s transporter (such as copper, calcium, zinc, manganese), and therefore can interfere with iron absorption and produce iron deficiency [4]. Moreover, multiple micronutrient deficiencies are likely to have a synergistic effect on the development of anemia, to which their contribution are linked to their frequency [4]. . Furthermore, despite the direct association with anemia, micronutrient deficiencies were reported to mediate the effect between nutritional anemia and other determinants. For instance, they were hypothesized to be associated with socioeconomic conditions, both through inadequate health care delivery, inadequate maternal-child practices and food insecurity [32]. In turn, nutrition (adequacy of dietary nutrients and dietary diversity) can intermediate the effect of both maternal-child practices and food insecurity on nutritional deficiencies (see figure 2) [32].

Anemia and infections

Infections can cause anemia indirectly through micronutrient deficiencies (by causing malabsorption of nutrients or anorexia), but also directly through hemoglobin loss (by blood loss, inflammation, hemolysis) [32, 34]. Hookworms (*Necator americanus* and *Ancylostoma duodenale*) and schistosomes (particularly *S. haematobium*) were reported to be the largest direct contributions to anemia through blood loss [1, 5]. For instance, estimates report that one half of the anemia moderate-to-severe cases in children may occur due to hookworm infestation [4]. Nevertheless, the intensity of infection, as well as coinfection with multiple parasites determine the severity of blood loss [4]. Additionally, schistosomiasis may also contribute to anemia through splenic sequestration of erythrocytes, increased hemolysis or anemia of chronic disease [4]. Besides of leading to a less severe blood loss, *Ascaris lumbricoides* infection also influence the iron status through gastric and intestinal ulceration and *Trichiura. trichiura*, may lead to iron deficiency by causing malnutrition and dysentery [14]. *Giardia lamblia* is also associated with malabsorption of micronutrients and in turn, malaria associates with severe anemia by causing acute and chronic hemolysis, subsequent suppression of erythropoiesis, and possibly secondary folate deficiency [4, 14, 35]. Because enteric nematodes, cestodes, trematodes and protozoans often occur sympatrically in endemic areas, polyparasitism has been suggested to have a synergistic effect in exacerbating detrimental health outcomes in infected individuals (see figure 3) [34, 36, 37]. Several other infections were also associated with anemia, namely viral and bacterial infections, but they will not be included in this study. It should be considered that in general, infections (mainly acute and chronic infections) can contribute to “anemia of chronic disease” or “anemia of chronic inflammation”, by stimulating the production of pro-inflammatory cytokines that alter iron metabolism (iron is sequestered and stored as ferritin), lowering the production and lifespan of red blood cells [4].

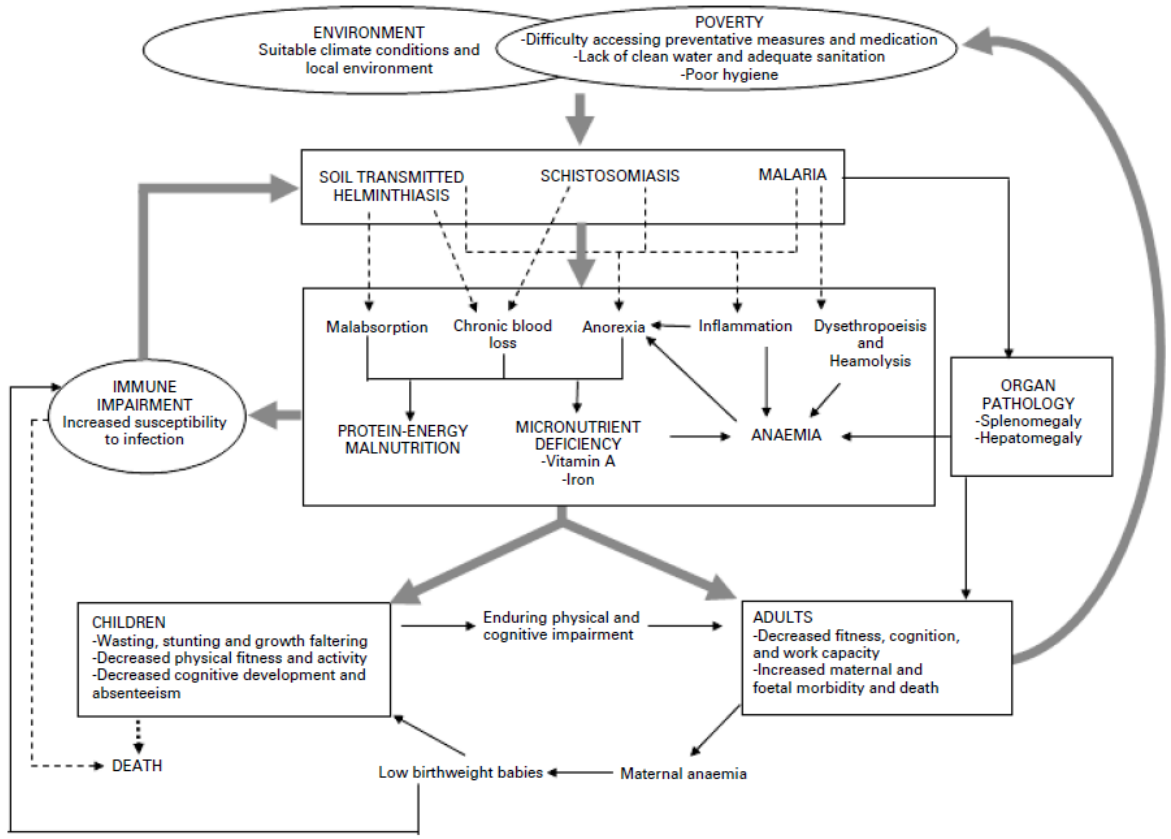

Figure 3 – Conceptual framework of the negative aspects associated with polyparasitism (Source: Pullan *et al*. 2008) [34].

It is also important to be kept in mind that, while some parasites are the cause of micronutrient deficiencies, the nutritional state of the host can also influence the establishment of the infection itself and influence the pathway by which they will lead to anemia. For instance, when facing a malaria infection, pre-established:

- Vitamin A deficiency may contribute to anemia by increasing the susceptibility to parasitemia, modulating iron metabolism and impairing immune function.
- Vitamin E deficiency may contribute to anemia by leading to oxidant damage and consequent hemolysis.
- Riboflavin deficiency by decreasing iron absorption, increasing erythrocyte fragility and affecting erythropoiesis.
- Folate deficiency may contribute to anemia by impairing erythropoiesis.
- Iron deficiency by leading to impaired erythrocyte synthesis.
- Zinc deficiency by impairing immune function, increasing parasitemia and leading to oxidative damage [35].

Interestingly, those micronutrient deficiencies can also protect against malarial anemia, specifically, vitamin E deficiency may protect from anemia by preventing antioxidant activity which increase the susceptibility of malaria parasites to oxygen radicals, while riboflavin deficiency may diminish parasite multiplication and growth, folate deficiency may impair the parasite metabolism, iron may impair parasite multiplication [35].

Recently, zinc was described to be a predictor of hemoglobin levels, and consequently associated with the occurrence of anemia, possibly contributing more to the development of anemia than iron itself [33, 38, 39]. During inflammatory events, zinc is confined into cellular compartments decreasing the concentrations of plasmatic zinc in an attempt to withhold it from the pathogen [40]. This nutritional immunity (a process by which a host organism sequesters trace minerals in an effort to limit pathogenicity during infection) was suggested to be mediated by inflammatory cytokines [41]. Furthermore, zinc it's among the trace elements whose metabolic profile change during both inflammation and infection [41]. Additionally, zinc deficiency was reported to lead to immune dysfunctions, that consequently lead to worse responses towards infections [41]. For instance, zinc deficiency was reported to account for near 18% of malaria cases, with zinc supplementation being associated with decreased prevalence of malaria [41, 42].

Genetic causes of anemia

The normal adult hemoglobin molecule is composed of four globin subunits (two *α* and two *β*), one heme (a red pigment molecule), including one iron (II) atom in each heme molecule. Furthermore, more than 1,000 human hemoglobin structural variants have been described in the literature, a number of which are also functionally abnormal [4, 14]. These abnormalities may result from mutation in the globin structural genes (with consequent qualitatively or quantitatively abnormal hemoglobin), or in globin regulatory genes (with consequent production of lower quantity of hemoglobin) [4]. One of the most frequent variants worldwide results from the mutation in the beta-globin gene that leads to the production of sickle cell hemoglobin, named hemoglobin S [14]. Generally, individuals with one hemoglobin S gene are phenotypically normal (sickle cell trait) and people who inherit two hemoglobin S genes have sickle cell (or Hb SS) disease (associated with low hemoglobin values (7 or 8 g/dL), vaso-occlusive crises, long term organ damage and decreased survival) [14]. On the other hand, quantitative alterations in globin gene expression may result in thalassemia (not to be further addressed here). Hereditary enzymopathies can also cause anemia [43-45]. One of the more prevalent worldwide is G6PD deficiency, where more than 300 genetic variants of the *G6PD* gene have been described [46-48]. However, most of the mutations are rare [49]. Hemolysis due to G6PD deficiency leads to decreased hemoglobin levels, and it is dependent on the class of the enzyme deficiency state and the magnitude of the oxidant impact [14].

Other determinants contributing to anemia

The prevalence and distribution of anemia (particularly IDA) is considered to have fundamental, underlying, intermediate and immediate determinants, involving a complex interplay of factors. i) Fundamental determinants involve socioeconomic, political, climatic and environmental conditions, ii) underlying determinants relate to agricultural factors (food/cash flow, crop yields, livestock), economic circumstances (regional/local wealth, equity/equality, literacy/education) and health policies (health coverage/insurance, control programs, fortification policies); iii) intermediate determinants relate to food availability (food security, household income/allocation/wealth, access to cereals/vegetables/meat, food fortification, access to fortified complementary foods, meal/dietary patterns) and health care (access to health care, access to supplementations, health worker knowledge, sanitation and hygiene); iv) immediate determinants relates to nutritional micronutrient intake (iron content of food, iron availability, heme-non/heme content, consumption of inhibitors and enhancers), blood loss due to infections and genetics [32, 50].

From a general perspective, socioeconomic, behavioral and environmental determinants can put some individuals and population groups at a higher risk of anemia [2, 32, 50]. Socioeconomic status affects the prevalence of anemia through several pathways. For instance, poverty is associated with poor housing, water, sanitation and hygiene, and inadequate infrastructure that may lead to increased disease risk, adverse nutrition behaviors (such as poor dietary practices, and poor dietary quality) and inadequate access to anemia prevention and treatment services (such as iron supplements, deworming and insecticide treated bed nets). Women and children in the lowest wealth quintiles were found to have 25% and 21% higher risk of anemia, than those in the highest wealth quintiles [4]. On the other hand, the low maternal education level may affect mother´s ability to access and understand health and nutrition information, negatively affecting their children's quality of diet, or influence decision-making and compliance with recommended health and caretaking practices, being frequently associated with anemia [12, 51, 52]. It has been also reported that some populations belonging to specific settings or ethnic, cultural or religious groups may be at a greater risk of anemia due to having less opportunities for generating income, accessing education, health care, social services, water, sanitation and hygiene, but also to being differentially exposed to infectious, having different dietary patterns or a different genetic background [4]. Gender inequality and cultural practices related to marriage and pregnancy (number and spacing) may also play a role in the development of anemia in children, whenever mothers are at increased physiological risk conditioned by the factors mentioned above, but also, households food-distribution practices or other dietary or feeding practices may lead to differential risk of anemia between boys and girls [4].

Regarding environmental factors, it should be considered that despite that the prevalence of anemia is generally estimated at the national or subnational level, it is known that it varies spatially at much

lower levels of aggregation, based on the spatial distribution of biological and socioeconomic factors associated with anemia.

Physiologic and biologic aspects associated with anemia

In turn, the proportion of anemia attributable to the nutritional, infectious and genetic causes discussed above may vary according to several physiologic and biologic aspects. In groups at high risk, multiple of these factors are frequently acting simultaneously to affect the risk of anemia. For instance, an imbalance between the nutrient needed for erythropoiesis and the higher nutrient demands for rapid growth, place under 5 years children in high-risk for developing IDA [4, 53, 54]. Healthy term infants are reported to be generally born with adequate iron stores who can last approximately 6 months, depending on maternal iron status during pregnancy [1, 14, 55]. It was reported that infants of anemic mothers were almost 6 times more likely to become anemic by the first year of life, even after adjusting for several confounding factors such as socioeconomic status, feeding practices, and morbidity [14]. However, while generally considered adequate for the first 4 to 6 months of life, it should be considered that the quantity of iron in breast milk may decrease through the course of breastfeeding period and may not be absorbed as efficiently as predicted [14, 56, 57]. Sequentially, the introduction of complementary foods, should have appropriate quantity and bioavailability of iron and low levels of inhibitors of iron absorption, who could reduce the absorption of iron from human milk [56, 58-60]. On the other hand, male infants may be at greater risk of lower hemoglobin concentrations and/or poor iron status as compared to females [4]. An assessment of iron stores in infants during the first year of life found that male infants had consistently lower iron stores and estimated body iron, and higher rates of iron deficiency than female infants [61, 62]. It was suggested a role of factors in utero, potentially hormonal influences, in determining iron status at birth and on erythropoietic activity [4].

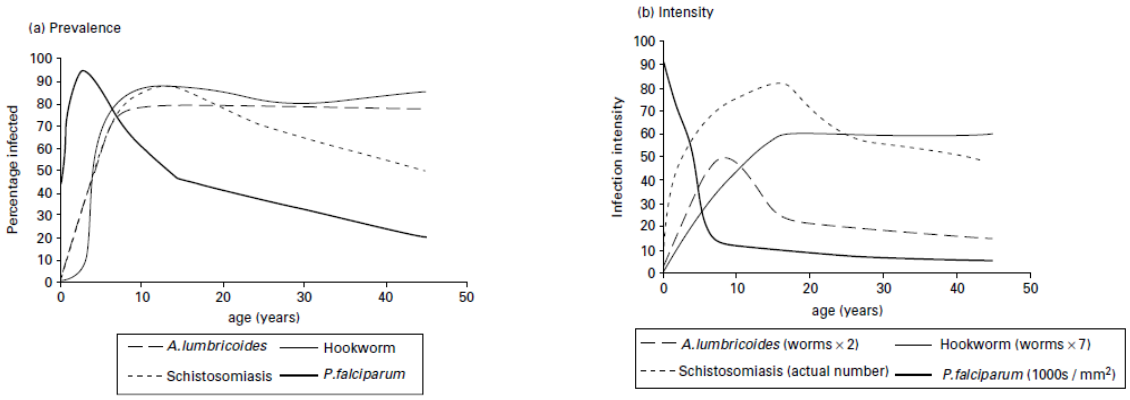

Figure 4 - Typical age profiles for the prevalence and intensity of infection by *A. lumbricoides*, *S. mansoni* and *P. falciparum* - adapted from Anderson and May (1991) (Anderson 1991).

Designing strategies to reduce anemia

Designing strategies to reduce anemia require previous situation analysis that includes determining the context-specific profile, composed by documented characterization of anemia frequency, spatial distribution, risk groups affected, the contribution of its determinants and possible opportunities and treatments. Those evidences must then sustain either a nutrition-specific approach, that is targeting mainly the immediate determinants of anemia, a nutrition-sensitive approach, which target fundamental/basic, underlying and intermediate transversal to a wide range of sectors, or a combination of both [4]. Thus, while defining the immediate determinants (nutritional, infectious and/or genetic) would allow designing short-term control strategies, and determining the relevance of fundamental/basic, underlying and intermediate determinants would allow designing medium-to-long term prevention strategies, a combination of both would comprehend both control and preventive dimensions and could tackle anemia more comprehensively [4, 50].

Nutrition-specific approaches

These types of interventions frequently aim at increasing/improving dietary diversity, bioavailability and intake of micronutrients, or by changing inadequate nutrition-related behaviors and practices (using social and behavior-change communication strategies) [4]. The common strategies to increase micronutrient intake and absorption are providing supplementation, adding micronutrients to the diet (fortification) or improving the quality and quality of diet through food-based strategies.

Supplements provide large number of essential vitamins and minerals in only few doses, being a very effective option for some populations and settings. However, programs adopting supplementation shouldn´t lost track of other nutritional causes of nutritional anemia [14]. Nutrient supplementation may only be efficacious in preventing and controlling anemia when it is caused by the target micronutrient deficiency, and when basic and underlying causes of deficiencies do not remain unaddressed. Unfortunately, if the full etiologic profile is not well described, IDA may be assumed as the primary cause of anemia and supplementation with iron may be inadequately considered the most suitable control strategy [14, 63, 64].

Mass fortification (addition of micronutrients to products consumed regularly by the general public) or target fortification (addition of micronutrients to products consumed by target subgroups) can also be equated for programs. Generally, they are well accepted, can be personalized to reach all sectors of the population, do not require the cooperation of the individual, the initial cost is relatively small, and the maintenance expense is lower than supplementation [14]. However, adding micronutrients to food require caution, mainly due to the possibility of excessive micronutrient intake, sensorial alterations, restriction on the foods that can be used and negative interactions between food components [65, 66].

Food-based strategies encompass a wide variety of interventions mainly aiming at 1) increasing the production, availability, and access to micronutrient rich foods, 2) increasing the consumption of micronutrient rich foods (improving either the overall quantity and quality of multiple erythropoietic micronutrient), 3) increasing the bioavailability of micronutrients in the diet (by increasing the consumption of enhancers and decreasing the consumption of inhibitors of nonheme iron absorption) or 4) combining some of those aspects [14]. Their overall goal is to empower individuals to improve the quality of their own diets [14, 67] [14, 50]. These approaches can be delivered through education, communication, social marketing, and behavior change programs to target specific groups [2, 4, 58, 67-77]. In general, two main strategies are frequently considered, namely dietary diversification and enhancement of micronutrients bioavailability and infant and young child feeding practices (either breastfeeding and complementary feeding), which will be further discussed here.

Dietary diversification and micronutrient bioavailability

Diets in rural areas from developing countries were reported to be based mainly in cereals, starchy roots and/or tubers and legumes, having normally less frequent consumption of dairy products and flesh foods (which are rich sources of readily available heme-iron, zinc and vitamin A as retinol, probably associated to disadvantaged dynamics of anemia-related determinants mentioned above [78, 79]. Plant-based diets are reported to have high content in phytic acid, dietary fibers and polyphenol, components that may inhibit the absorption of important trace minerals (mainly non-heme iron and zinc), and unsurprisingly, that pattern was reported not to provide the daily needs of calcium, iron and zinc, as observed by Gibson *et al* 1998 in complementary foods used in some parts of Africa [80-85]. Additionally, they frequently have low fat content, which further limit the absorption of lipid-soluble (pro)vitamins, e.g., provitamin A carotenoids. When considering dietary diversification to tackle micronutrient deficiency in those settings, it should be considered that the simultaneous consumption of non-heme iron rich-foods, and enhancers of iron absorption (citric, malic or ascorbic acid) or heme-iron rich foods (such as meat, poultry or fish) may improve iron absorption by limiting the effect of iron inhibitors [86]. Furthermore, it should also be taken into account that the consumption of legumes, green leafy vegetables, whole grains and fruits/fruit juices may potentially increase folate levels naturally, the consumption of green leafy vegetables, orange and/or yellow fruits and vegetables, dairy products, eggs and fish may increase vitamin A levels, and animal-source foods (such as shellfish, meats, fish, poultry), and dairy products may increase the levels of Vitamin B12 [4]. Furthermore, some methods of food processing (soaking, fermentation, germination or thermal or mechanical processing) may also alter the bioavailability and absorption of micronutrients [4, 81].

<u>Nutrition-sensitive approaches (infection-oriented)</u>

These approaches address mainly underlying and basic causes of anemia from a wide range of sectors, including disease control, WASH and intersectoral strategies that address root causes (for example poverty, lack of education and gender norms). The control of parasitic infections aim both at stopping transmission (by treating infection) and also at reducing the morbidity that they cause (such as anemia). In order to achieve the nutrition-sensitive approach aims, generally three main strategies are adopted:1) pathogen-targeted chemotherapy that can bring direct relief from disease, promote child development, and help slow transmission; 2) long term programs such as vector control, Water, Sanitation and Hygiene (WASH) measures, or health education to change behavior that will help to prevent transmission; 3) a combination of both strategies [2, 87, 88]. The plausibility of those interventions is based on the assumption that in poor settings, where high prevalence, intensity, reinfection, incidence and co-endemicity of anemia-related infections occur, there is an important contribution of micronutrient deficiency anemia related to infections, potentially refractory to supplementation and requiring multicausal resolution approaches [2, 20, 89-92]. Besides, in setting with a highly infectious profile, the screening and treatment of infections must be provided (especially in the case of malaria), in order to further prevent supplementation to adversely affect the infection-associated morbidity and mortality in children [50, 93-97].

One of the first infection to be considered is malaria, responsible for about 35% of anemia in African children, and whose control in endemic areas was reported to greatly reduce anemia and severe anemia (by over 25% and by 60%, respectively) [4, 20, 22, 98-102]. When prevention is to be considered, chemoprevention and vector control are frequently planned. Intermittent preventive treatment comprehends the administration of sulfoxide–pyrimethamine, designed to protect children against clinical malaria and anemia in the first year of life, while vector control is the key intervention for global malaria control, aiming mainly at increasing the use of long-lasting insecticidal nets and indoor residual spraying to decrease transmission [2, 4, 98].

Soil-transmitted helminths, schistosomiasis and environmental/tropical enteropathy (caused by oral-fecal contamination or contact with contaminated water and soil), should also be considered[4, 52, 103-105]. Therapeutic approaches, aiming at reducing the burden of soil-transmitted helminths (STH) infections and the intensity of infections, include single-dose, large-scale mass deworming, periodic preventive chemotherapy, or repeated large-scale administration of anthelminthic drugs to at-risk populations [106, 107]. For schistosomiasis, control includes large-scale drug treatment of at-risk populations (with praziquantel), snail control, improved sanitation, and health and hygiene education. Furthermore, multisectoral strategies, targeting adequate water, sanitation and hygiene practices may maximize and sustain the benefits of a decreased burden of those infections. For instance, promoting improvements in key hygiene behaviors (such as hand washing), treatment and use of safe water,

and using hygienic methods of disposal of feces and urine can be incorporated into health-sector activities, such as nutrition counselling, promotion and assessments, as well as health visits.

The effect of combining strategies to reduce anemia

Integrating therapeutic (deworming) and preventive (either food-based or WASH/malaria education) strategies can simultaneously treat infections and increase hematopoietic micronutrient intake or reduce disease transmission, which could result in the reduced anemia [2, 67-75, 90, 106-112]. For instance, deworming plus nutrition education has been reported to reduce anemia from 82.0 to 55.4%, while increasing green leafy vegetables consumption from 44.7 to 60.6% [75]. Furthermore, exclusive educational interventions targeting nutrition improvement, increased mothers ability to identify malnutrition (from 15% to 99%), exclusive breastfeeding (79% versus 48% in control), weight gain (0.86kg versus 0.77kg in control), vegetables feeding, nutrient-dense foods at lunch (11% of difference between intervention and control groups), dietary requirements for energy, iron and zinc, complementary feeding, and significantly reduced rates of stunting [68-72, 76, 113]. On the other hand, exclusive WASH educational interventions, health education on soil transmitted helminths and schistosomiasis, significantly increased knowledge, reduced the prevalence of *A. lumbricoides* and *Schistosoma mansoni*, reduced the incidence of hookworms and the intensity of trichuriasis, ascaridiasis and hookworms [107, 108]. When deworming is added to WASH education, prevalence and intensity of soil transmitted helminths decrease and school children scored higher than control on their knowledge [106, 108]. Studies evaluating educational approaches to malaria prevention showed improved knowledge regarding breeding sites, bed-net use and indoor spraying, increased windows and door net use and practice of maintaining clean environment and, a reduced number of reported malaria episodes and fever incidence, [109, 110, 114, 115].

As mentioned, several comprehensive intervention strategies have been tested. However, they frequently evaluate the impact of education on nutrition or on infectious etiologies (whether or not combined with drug therapy) separately [68-75, 106-111]. Thus, differences in their designs and methodologies hinder the face to face comparison and evaluation of results, rendering it difficult to define the best approach to reduce anemia. For instance, differences on the target population (adult or children), dimensions of the learning package (e.g. for sanitation: stool disposal, water quality or supply), the deliverers (community promoters/volunteers, teachers, local health workers, community leaders), the place where education occurs (at health center or at the communities), the number of intervention contacts, type of contacts (group meetings and/or individual contacts), duration of the intervention/observation and mainly the combination with other strategies (micronutrient supplementation, construction of latrines and hand-washing mechanisms, etc.) can be observed [68-75, 106-111]. Furthermore, one of the most inclusive studies, published recently by Humphrey *et al*.

2019, investigated the effect of two WASH interventions, either alone or combined with improved Infant and Young Child Feeding (IYCF) practices, concluding that education in nutrition reduce anemia and , but adding WASH to the intervention had no major improvements on those effects [116]. Similar results were reported by Null *et al*. 2018 within a cluster-randomized trial using interventive groups with several WASH and nutrition combinations [117]. Neither study investigated the effect of combining deworming, nor was the impact of malaria preventive actions included.

As mentioned, in the Bengo province of Angola, malaria and schistosomiasis were reported to be responsible for 16% and 10% of anemia cases, while ascariasis was associated with undernutrition (in turn responsible for 13% of anemia cases) [20, 22]. Furthermore, between 2012-2013, Lemos *et al*, tested a therapeutic intervention in children (with 2-15 years) from a highly *S. haematobium* endemic setting of that province [21]. The authors reported low effectiveness in reducing infections when a mass administration of praziquantel was integrated with albendazole and with an antimalarial test-and-treat [21]. They described that prevalence reductions one month after treatment were relevant for urogenital schistosomiasis and intestinal parasites, but that 6 months later the prevalence's were similar to the ones at the baseline [21]. Additionally, the variation in the prevalence of *P. falciparum* was postulated to have been biased by the seasonality of malaria (which increased one month after treatment but decreased six months after), while the very high prevalence of anemia was observed to have non-statistical reduction [21]. Those results reiterate the high prevalence of infections and anemia in Angola and suggest that the beneficial effect of exclusive therapeutic interventions may not be sustained in our setting, possibly due to heavy contaminated environments. As far as we know, there is no effectiveness results regarding nutrition-specific interventions in the country.

Aim

The nutrition-specific approaches commonly aim at increasing/improving dietary diversity, bioavailability and intake of micronutrients, or changing inadequate nutrition-related behaviors and practices. This can be sustainably achieved through food-based strategies by educating and empowering caretakers to improve the quality of their children's diets, which in turn could result in improved overall quantity and quality of multiple erythropoietic micronutrients, increased consumption of enhancers and decreased consumption of inhibitors of iron absorption and as a consequence, increased hemoglobin level and reduced anemia, micronutrient deficiency and malnutrition prevalence. On the other hand, nutrition-sensitive approaches (infection-oriented) address mainly underlying and basic causes of anemia from a wide range of sectors, including disease control, water, sanitation and hygiene and intersectoral strategies that address root causes. The control of parasitic infections aims both at stopping transmission (by treating infection) and also at reducing the morbidity that they cause (such as anemia), for which the main strategies adopted are pathogen-targeted chemotherapy, long term programs such as vector control, water, sanitation and hygiene (WASH) measures, or health education to change behavior that will help to prevent transmission.

Integrating therapeutic (deworming) and preventive (either food-based or WASH/Malaria education) strategies can simultaneously treat infections and increase hematopoietic micronutrient intake or reduce disease transmission, which could result in the reduction of malnutrition and anemia. For that effect, several comprehensive intervention strategies have correspondingly been tested. However, they frequently evaluate the impact of education on nutrition or on infectious etiologies (whether or not combined with drug therapy) separately, thus presenting differences in their designs and methodologies and rendering it difficult to compare their results and define the best approach to reduce anemia.

In this study, we aim at comparing the effectiveness of two educational community-based interventions in nutrition and in WASH/malaria education, both combined with a test-and-treat therapeutic approach, for the reduction of anemia in preschool children. In the process, we aim at describing the dynamics of anemia in African children population, to measure the prevalence of anemia and its etiologies, to describe the main factors separately associated with the occurrence of Iron Deficiency Anemia (IDA) and non-IDA anemia, to investigate if zinc deficiency is directly associated with anemia or if it relates (interact or mediate) with inflammation or infections in the association with anemia and to determine the effectiveness of adding an educational intervention in WASH/Malaria in reducing nutritional anemia, when compared with an educational intervention in nutrition.

Study setting

CISA´s 4,700km^2 study area, located approximately 75 km East of Luanda, in the Bengo Province, comprehend 5 communes from the Dande municipality in the Bengo Province (Úcua, Caxito, Mabubas, as well as some hamlets from Kikabo and Barra do Dande). The demographic and economic aspects of their 15,579 households and 59,635 residents (registered initially) are being followed since 2009 and where several studies have been conducted [20, 22, 118-124]. However, the entire extent of the commune population resident in Kikabo and Barra do Dande are not fully surveyed by the Dande´s Health and Demographic Surveillance System (HDSS) and their hamlets were not considered eligible for study. Furthermore, despite that 15 hamlets with health facilities were registered within the 3 communes, only 7 were found to provide daily primary care and were included in this study [125]. Those hamlets were Paranhos, Riceno, Sassa Povoação, Boa Esperança, Porto Quipiri and Caboxa.

General characteristics of the study

This was an open (unblinded), parallel, cluster-randomized controlled effectiveness trial, with 3 study arms: control, WASH/Malaria and Nutrition, implemented over a 12-month follow up. In sum, two operational moments were implemented; 1) the evaluation moments (baseline, 6 and 12-month follow up waves) that aimed at collecting the impact indicators and deliver the test-and-treat therapeutic component of interventions; and 2) the educational moments that aimed at improving the quality of target parental practices with six domiciliary counselling visits and collecting the process indicators (see next figure). Thus, determining the effectiveness of interventions was based in a “by protocol” statistical analysis, that required determining the frequency of anemia and its etiologies, the determinants associated with Iron Deficiency Anemia (IDA), evaluating longitudinal variations in the primary and secondary outcomes in each of the study arms (green arrow) and comparing those variations between study arms (yellow arrows in next figure).

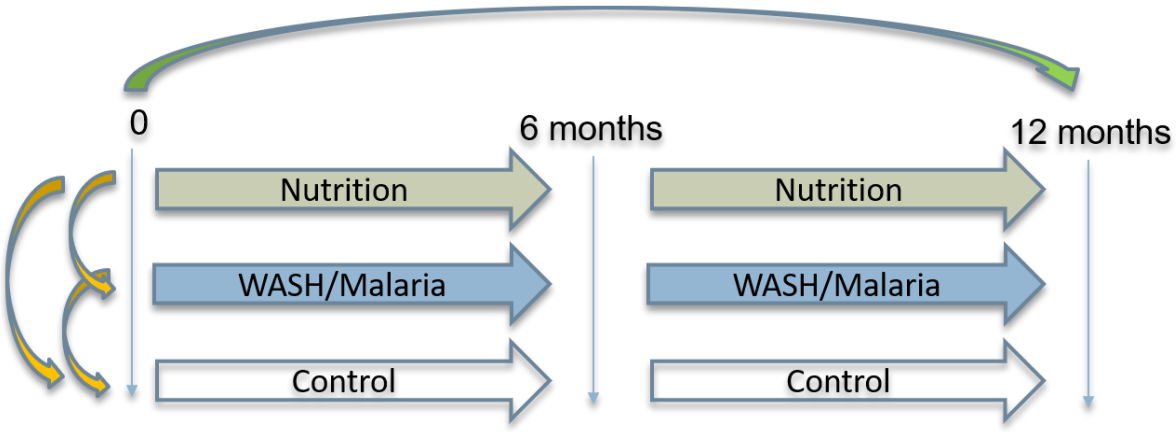

Figure 5 – General study design

Sampling strategy

In this study, the chosen sampling strategy was a non-probabilistic (convenient) sampling. Hamlets with functional health posts were chosen due to the greater facility in recruiting and mobilising the population and corresponding logistical advantages. The census approach was adopted because variations in the density of eligible children were expected, upon estimations from the Health Research Center of Angola (CISA, translated) HDSS database information (24th October, 2014), and the real density in each cluster was needed.

Participants, eligibility and follow up

Within those hamlets, we used a door-to-door approach (census approach) to list all under-3-year-old resident children and their mothers/caregivers, explain the study and invite them to participate at the baseline evaluation being conducted at the health center in the following days. This age group was selected because children are expected to have limited mobility, generally receive higher parental attention, and have their exposure to contaminated environments more controllable, when compared to school-aged children. Children were not recruited whenever they received blood transfusions recently, report adverse reactions to albendazole and/or to praziquantel, or if their caretakers did not commit to completing both the evaluation and the educational moments of the study. At the end of the census approach, 1,106 households were found to be eligible and invited to participate, from which only 830 caregivers attended, and 949 children were evaluated.

From those, one children was found to be older than 3 years old and other had no hemoglobin data and both where excluded from the baseline statistical analysis. Furthermore, from the 947 children enrolled at baseline, 6 were excluded and 942 were randomized to the interventions (see figure 6). From those, 150 were lost during the 12-month intervention period, defining a dropout rate of 15.9%

(from which 2.7% (4/150) occurred in the Control group, 50.7% (76/150) in the Nutrition group and 46.7% (70/150) in the WASH/Malaria group). These dropouts were observed to be differential between groups regarding the mean of zinc levels (p-value=0.042. Also, 151 participants were excluded from the effectiveness analysis due to absence of 12-month follow up wave data. These participants were not considered to be dropouts because they were later evaluated in other follow up waves (within another study). Nevertheless, they lead to substantial denominator variations.

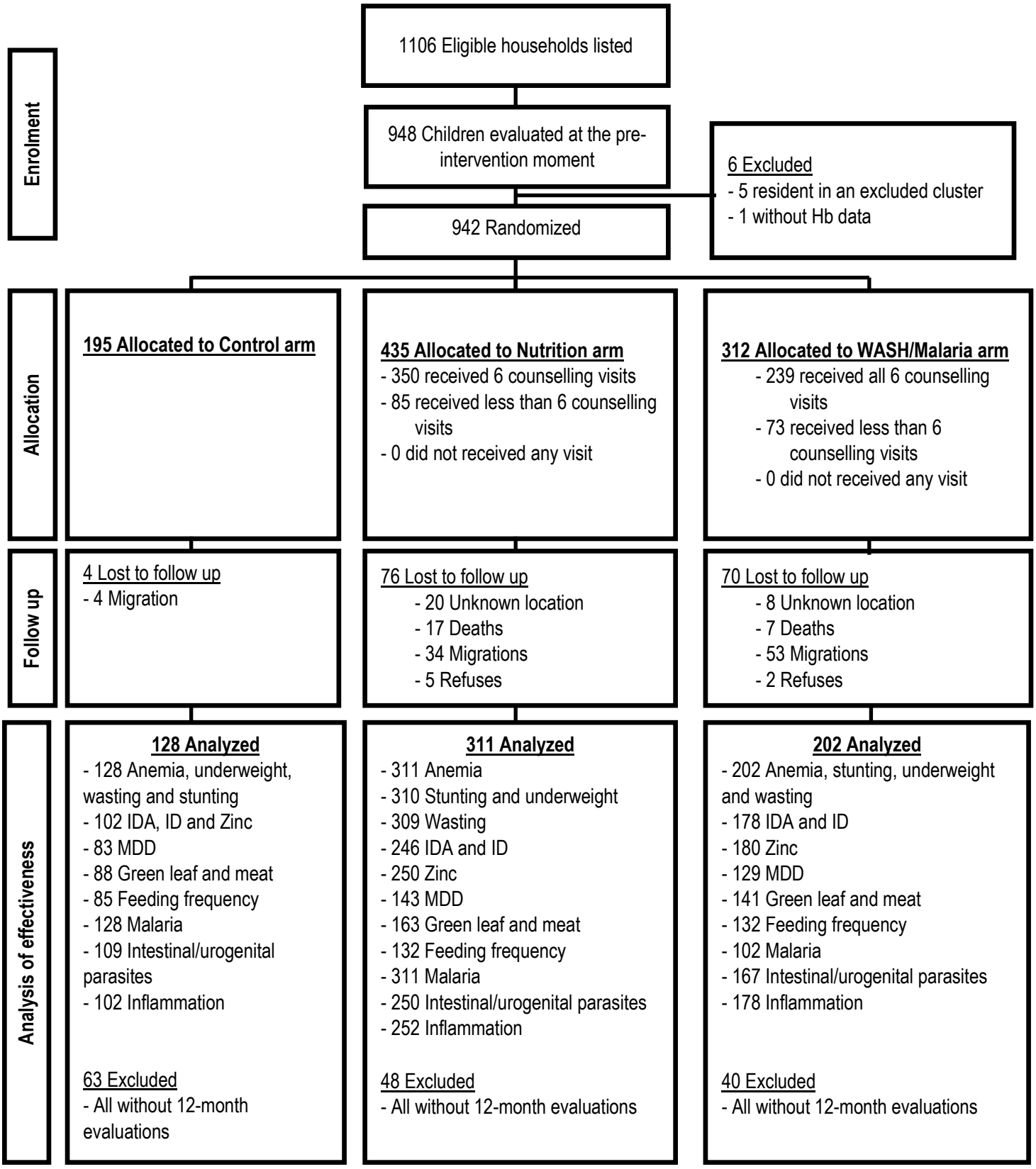

Figure 6 – Children follow up flowchart. Hb – Hemoglobin, IDA – Iron Deficiency Anemia, ID – Iron Deficiency, MDD – Minimum Dietary Diversity.

Recruitment and mobilization

Baseline

A house-to-house recruitment team identified the children eligible in each hamlet, explaining the importance of the study both to local authorities and caretakers, delivering stool and urine containers and explaining the best practices for sample collection. This team was instructed to recruit 45 children per day and to orient them to attend the next evaluation day at the hamlet health post. Those evaluations were carried out in each hamlet until all eligible willing-to-participate families have been notified.

6 and 12-month follow up wave

To revisit families, field technicians performed a house-to-house invitation strategy, using Dande´s HDSS household identification system, reference points and family phone numbers. To increase adherence and promote participant retention, the hamlet coordinators and/or traditional authorities were invited to be present at all phases of the study. Furthermore, incentive kits were distributed to all children during the follow up moments, namely a nutritional kit (water and yogurt) and a WASH/Malaria kit (soap and lye).

Hypotheses under study

Nutritional group

The Nutrition arm, assumes that the test-and-treat approach would clear infections and that the education in nutrition would improve the knowledge and awareness of caretakers as regards the causes, prevention and treatment of nutritional anemia, which in turn may reflect in behavioural changes that reduce the prevalence of inadequate breastfeeding and child-feeding practices. This could in turn improve dietary diversity, by increasing the consumption of erythropoietic-rich foods (iron, vitamin B12, vitamin A and folate) and enhancers of iron absorption (vitamin C), while decreasing the inadequate consumption of iron absorption inhibitors (polyphenols, phytates, calcium, etcetera)) [75, 76, 126], ultimately leading to the decrease in the prevalence of nutritional anemia.

WASH/Malaria group

The WASH/malaria arm, assumes both that the test-and-treat approach clears infections, and that education in appropriate water, sanitation, personal hygiene and malaria prevention practices improves the knowledge of caretakers regarding the causes and preventive measures against infections, which in turn would lead to behavioural changes, and consequently to reduced prevalence, intensity, incidence and re-infection rates of those diseases [106, 114, 127-129]. Thus, considering that parasitic infections are important causes of anemia within this setting, this intervention would consequently decrease the occurrence of infection-related anemia.

Control group

The Control arm, assumes that an exclusive biannual test-and-treat approach will clear the anemia-related infections and that in consequence the levels of hemoglobin will increase and the prevalence of anemia will decrease.

Interventional package delivered

Families/caretakers allocated to the interventions received six personalized counselling sessions at their homes. The overall treatment given in each arm, throughout the duration is described in table 1.

Table 1. Summary of the treatment predicted to each arm.

	Nutrition arm:	WASH arm:	Control arm
Diagnosis and treatment of: - Malaria (Artemether-Lumefantrine 20/120), - Schistosomiasis (Praziquantel) - Intestinal parasites (Albendazol and Tinidazol)	Provided	Provided	Provided
Distribution of bednets at the baseline	Provided	Provided	Provided
Distribution of soap and lye at the first and second follow ups	Provided	Provided	Provided
Personalized, home-based counseling of primary caretakers	Provided	Provided	Not provided
Targets within the educational package Each visit addressed 2 behaviors of the specified targets topics, moving to the next pair in later visits	1) Breastfeeding; 2) Complementary feeding*; 3) Weekly adequate food diversity; 4) Appropriate number of meals; 5) Adequate amount of food; 6) Responsible feeding; 7) Disease and alimentation; 8) Hygiene and food safety.	1) Bednet usage; 2) Reducing mosquito breeding sites 3) Prevention of open sky defecation; 4) Latrine cleaning; 5) Adequate hand washing; 6) Healthy backyard environment; 7) Adequate: water availability, treatment, transportation and storage; 8) Adequate personal hygiene.	Not applicable
Number of counseling visits	6	6	Not applicable
Total duration of the follow up	12 months	12 months	12 months

Illustrative materials, adapted from UNICEF recommended approaches were used for these counselling sessions [130, 131].

Randomization

The hamlets were considered as cluster units due to their administrative and geographic isolation characteristic, in order to decrease the possibility of cross contamination between individuals in different interventional arms living in the same hamlet (that could happen if individuals were chosen

as sampling units). For randomization, the names of the hamlets were written down on pieces of paper and placed in a bag and then successively removed. The first two papers to be removed were attributed to the Nutrition arm, the following two to the WASH/Malaria arm and the next pair to the control, the procedure repeated until there are no papers left in the bag. The randomization was carried out by the Study Coordinators. Only children with pre-intervention evaluation were allocated to the Randomized Controlled Trial (RCT).

Training

At the beginning of the study, 6 field workers and 2 nursing technicians were selected from the study area and trained. The one-week training have incorporated: 1) an introduction to the research questions, goals and study design, 2) basic concepts regarding the diseases studied, 3) methodologies for data collection (structured interviews, anthropometric evaluations, recognition of signs and symptoms of micronutrient deficiency, measurement of temperature) and 4) communication skills and the collection/delivery of information. Additionally, nursing technicians undergone 3-day training on: 1) the treatment and referral protocol adopted in this study, 2) best practices for drug administration in young children and 3) the management of domiciliary and health unit treatments. Blood, urine and stool samples were collected before their processing by experienced CISA resident laboratory technicians. A field work simulation class was conducted with all personnel involved in this survey at the end of all training sessions.

Providers

Field and nursing technicians, involved in the evaluation (baseline and follow up waves), were attributed to the different intervention arms, being responsible for the counselling sessions (monitoring, encouraging and continuously promoting behavioural change), under the direction of a supervisor. Their previous training of the providers was reinforced with theoretical and practical sessions on 1) the core aspects of the target disease and conditions (specifically, “What is the disease/condition”, “What are the signs and symptoms”, “What is causing them”, “What can mothers do to prevent them”), 2) counselling techniques (evaluating the emotional state of the caretaker and adapting counselling techniques to the emotional state), 3) household environment and risk behaviour evaluation, according to the respective interventional arm they belong to. Within each intervention arm, they have performed one cycle of three-monthly visits to a group of households before then moving onto another group of families. Following the first training stage, refresher sessions have occurred before the follow up evaluation moments and before every domiciliary personalized counselling. At the beginning and at the end of each training moment, technicians have undertaken a written evaluation test. Additionally, 20% of all counselling sessions (per technician) were subject to evaluation by a supervisor.

Receivers

The receivers, i.e, the targets for the behaviour change interventions, are mothers and/or primary caretakers of the children studied. After the baseline assessment, the mothers in each intervention arms were trained according to the respective “targets within the educational package” described in table 1. The objective is to empower mothers with knowledge on basic principles regarding: 1) anemia and its nutrition-related etiologies or 2) anemia and its infections-related etiologies (in particular, what are the signs and symptoms? How did the child become at risk? How can mothers prevent this and what they should do whenever their children fall sick?) and to stimulate inadequate mother-to-child behaviour changes. Technicians have documented pre-and post-intervention alterations in the household environment and mother-to-child practices (according to the process indicators described in table 2).

Data collection and processing

Sample and data collection at the Baseline and follow up waves

Questionnaire

Adapted from previously validated questionnaires [132, 133]. Aim at collecting Demographics (children and caretakers age and sex, number and age of other household residents), Socio-economic (household monthly income, daily food expenditure, water daily expenditure, latrine, bednet, land for agriculture and cattle ownership), Parental practices data on Water Sanitation and Hygiene (WASH), malaria prevention and Feeding practices.

Anthropometry

Weight was collected in a pediatric electronic or platform Scale, while height/length was measured using a harness or adult stadiometer, and mid-arm circumference was collected using a brachial metric tape. Those measures were used to calculate anthropometric indices of children’s and mothers for the diagnosis of undernutrition, according to WHO standards [134, 135].

Phlebotomy

About 1 ml of peripheral blood was collected on site by experienced health professionals, according to WHO guidelines to good phlebotomy practice [136]. The blood samples for iron, zinc and CRP determination were collected into Micro tubes 1,1ml Z-Gel® (Sarstedt, Nümbrecht, Germain), then centrifuged to separate serum, which in turn was stored at -20ºC until processing. Blood samples for molecular genetic analysis were collected on filter paper, air dried and stored until processing.

Stool and urine collection

Two sample containers were provided domiciliary for each child at the moment of recruitment or follow up mobilization. Caretakers were instructed to collect the stool in the day before the evaluation (following the instructions within the instructive flayer), using the spatula incorporated at the container and to store it in a cool place without direct light. Urine samples were advised to be collected at the second container within the same day of the evaluation. Children failing to delivery sample were encouraged to do so, within the same day or until all the hamlet was evaluated. A pediatric urine collection bag was applied to young children (incapable to verbalize urge to urinate). Formalin (10%) was added to stool samples, and along with urine samples, were stored in a thermal box with coolers for transportation to the lab and placed in the freezer until parasitological analysis was performed.

Laboratorial analyses within the Baseline and follow up waves

Parasitological analysis

- Diagnosis of *P. falciparum* and *Plasmodium vivax* malaria using Rapid Diagnostic Test

The screening of *P. falciparum* and *P. vivax* was conducted *in situ*, using a Rapid Diagnostic Test (*SD BIOLINE* Malaria Ag *P.f/P.v*® (Standard Diagnostics, Inc., Republic of Korea) performed according to the manufacturer guidelines .

- Diagnosis of *Plasmodium spp*. using Giemsa stained blood films

After being collected by venous puncture, the blood was placed into one slide and thick and thin blood films were performed, fixed, stained and read as described in the WHO´s Basic Laboratory Methods in Clinical Parasitology report [137].

- Screening of intestinal parasites using the Kato-Katz technique

This technique is indicated for the research of eggs of intestinal parasites (not being indicated for larvae or cysts). It is based on the filtration and clarification of stool with a solution of malachite-glycerin green, which facilitates the visualization of the eggs present in the preparation. Two smears for each sample were performed and read by 2 different technicians. Disagreements on positivity between the 2 observations were solved by a third technician whenever possible, if not, positivity was considered when parasites were found in at least one of the smears. Parasites identified with an unspecific technique was not considered.

- Screening of intestinal parasites using Parasitrap® kits (Biosepar, Germany))

This technique is based on the principle of centrifugal-sedimentation of parasite structures in formaldehyde and ether (Richie modified technique). It is indicated for eggs of helminths (light and heavy) and cysts of protozoa. Part of the stool samples within the initial stool container were transferred into the processing container (containing fixative solution to preserve eggs, larvae, and

cysts) and were stored until processing. A single examination per sample was conducted using this method. This method does not allow the determination of the intensity of the infection.

- Diagnosis of *S. haematobium* using a Syringe filtration technique.

This technique was performed as described in the WHO´s Basic Laboratory Methods in Clinical Parasitology report, using Whatman® Nuclepore™ membranes (diam. 25 mm, pore size 12 µm, polycarbonate, Merck, Germany) and the intensity of infection was classified according to the WHO´s Prevention and Control of Schistosomiasis and Soil-transmitted Helminthiasis report [137-140]. A single examination per sample was conducted using this method.

Biochemical analysis

- Determination of blood levels of hemoglobin (Hb)

Blood levels of hemoglobin were determined by imunocromatography, using an Hemocue® Hb 301 System (Angelholm, Sweden) photometer, according to the manufacturer. A single examination per sample was conducted using this method. In sum, the hemoglobin is converted to hemiglobin, and from this to hemiglobinazide when 10µl of whole blood is inserted into a microcuvette containing sodium nitrite and sodium azide. The absorbance of the final product of oxidation is read at 565 and 880nm, adjusting for presenting the concentration in gram of Hb present in 1dL of blood [141].

- Determination of serum levels of C-Reactive protein (CRP) and Ferritin

Determination of serum levels of C-Reactive protein (CRP) and Ferritin was performed using an automated autoanalizer (BT1500, Biotecnica Instruments S.p.A, Rome) and CRP turbidimetric latex® and Ferritin® kits from Quimica Clínica Aplicada (QCA S.A., Tarragona, Spain), according to the manufacturer [142].This is a method that allows the quantification by comparing the turbidimetric response produced by these compounds in the participant´s serum with the response of these compounds in samples with known concentration. A single examination per sample was conducted using this method, unless unusual values were observed.

- Determination of serum levels of Zinc by Colorimetry

Determination of serum levels of Zinc using an automated autoanalizer (BT1500, Biotecnica Instruments S.p.A, Rome) and Zinc® kits from Quimica Clínica Aplicada (QCA. S.A., Tarragona, Espanha). This is a method that allows the quantification of zinc by reading the color intensity produced by the zinc and dissodic salt chelation product (formed in alkaline solution), in a spectrophotometer [32]. Concentration is proportional to the color produced. A single examination per sample was conducted using this method, unless unusual values were observed.

Molecular genetic analyses

- DNA extraction

DNA extraction was performed using InstaGene™ Matrix (Bio-Rad laboratories, Inc. United States of América), according to manufacturer.

- Screening for sickle cell trait/anemia by PCR-RFLP

The determination of the sickle cell AA, AS and SS genotypes was performed by PCR-RFLP. In sum, the target fragments were amplified in a 20 µl reaction mixture containing 10µl of 2x MyTaqTM MixBIO, 1 µl primers (Primer 1_FW (10µM): ACCTCACCCTGTGGAGCCAC and Primer 67_RV (10µM): ACCAGCAGCCTAAGGGTGGGAAAATACACC), 7µl of water and 1µl extracted DNA. The real Time PCR, using a CFC connect Real-Time PCR Detection System (BIO-RAD), was conducted with a 5 min of the initial step at 94°C, 28 cycles of 1 min at 94°C, 65°C and 72°C, and 10min at 72°C program. Afterwards, 15µl of the amplification product was digested with 1µl of Bsu 36I enzyme (in a solution with 4µl of water). Electrophoresis to separate the fragments was performed in an 2% agarose matrix gel.

- Screening for G6PD deficiency by Real Time PCR [43, 143].

We used Real Time PCR to screen for the M98V (G202A) and N156D (A376G) Mis-sense Mutations within in genes located in the long arm of the X chromosome (Xq28), following the protocol published by Brito et al 2014 [43].

Data collection at the Educational moments

Questionnaire

Caretakers participating in the intervention arms had a monitoring file (consisting of six structured questionnaires (one for each monthly visit), applied by the technician to document behavioural changes at every counselling visit. The main data collected in each educational arm is described above:

Variables, outcomes and cut-offs

Baseline and follow up waves

Total anemia was classified as hemoglobin levels (Hb) below 11.0 g/dL, with the following stratification: mild anemia if Hb was between 10.0 and 10.9 g/dL, moderate anemia if Hb was between 7.0 and 9.9 g/dL and severe anemia if Hb was lower than 7.0 g/dL [4, 92, 144]. Furthermore, inflammation was classified as C-Reactive Protein (CRP) serum levels higher than 5 mg/L and children were considered to have Iron deficiency whenever serum ferritin was below 12µg/L (without

inflammation), or below 30µg/L (with inflammation) [145]. The classification of Iron Deficiency Anamia resulted from the combination of the variables described previously, in sum Hb level below 11,0g/dL in the presence of iron deficiency. Pathological zinc levels were considered whenever, serum levels were bellow 70.0 µg/dL (Zinc deficiency) or above 150.0 µg/dL (Zinc overload) [146]. The prevalence´s of helminths and protozoa were determined as the proportion between infected children and those delivering the correspondent sample. Children were considered to have diarrhea if caretakers reported that the children had 3 or more aqueous dejections per day in the last 2 weeks. Z- scores of weight-for- age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ) were determined using WHO Anthro software (version 3.2.2) for children and body mass index (BMI) was calculated and used to classify undernutrition in their mothers [147]. Exclusive breastfeeding under six months, continued breast feeding for children over 5 months, Individual Minimum Dietary Diversity, and consumption of iron-rich foods were classified as previously described, using 24h recall data [77, 133].

Educational moments

Primary outcomes comprised only health indicators, namely increase in hemoglobin levels and reduction of anemia prevalence, as described by Fançony et al 2019 [148]. Secondary outcomes comprised health and behavior indicators for the estimation of impact evaluations, while process indicators (which comprised either reported or observed practices or behaviors collected at the counselling visits) were used to conduct process evaluations. The complete list of outcomes and their analysis strategy within each arm is presented table 2.

Table 2 – Outcomes and analysis performed in this study.

Variables	**WASH/ Malaria**	**Nutrition**	**Control**
1) Primary outcome: *1.1) Impact on health measured at the evaluation moments**			
Hemoglobin levels (g/dl)	x	x	x
Anemia prevalence (%)	x	x	x
2) Secondary outcomes: *2.1) Impact on health measured at the evaluation moments**			
Iron deficiency anemia (%)	x	x	x
Weight (kg)	x	x	x
Stunting (%)	x	x	x
Underweight (%)	x	x	x
Having at least one intestinal/urogenital parasite (%)	x	x	x
P. falciparum (%)	x	x	x
*2.2) Impact on behaviour reported at evaluation moments**			
Minimum dietary diversity (MDD) (%)	x	x	x
Green leaf consumption (in previous 24h) (%)	x	x	x
Meat consumption (in previous 24h) (%)	x	x	x
Children sleeping under bednets in the previous night (%)	x	x	x
*2.3) Impact on behaviour observed by the technician at the WASH/Malaria educational sessions***			

Nº of domiciliary counselling visits delivered (%)	x	NA	NA
Having bednet in the children's bed (%)	x	NA	NA
Latrine ownership (%)	x	NA	NA
Having water to wash hands in the latrine (%)	x	NA	NA
Clean latrine (%)	x	NA	NA
Backyard environment with garbage (%)	x	NA	NA
Backyard environment with loose animals (%)	x	NA	NA
Backyard environment with still water (%)	x	NA	NA
Caretaker having clean nails (%)	x	NA	NA
2.4) *Impact on behaviour: reported by caretakers at the nutrition educational sessions***			
Nº of domiciliary counselling visits delivered (%)	NA	x	NA
Weekly cereal consumption (%)	NA	x	NA
Weekly seeds consumption (%)	NA	x	NA
Weekly Milk and derivatives consumption (%)	NA	x	NA
Weekly Food from animal sources consumption (%)	NA	x	NA
Weekly Eggs consumption (%)	NA	x	NA
Weekly Legumes consumption (%)	NA	x	NA
Weekly Vegetables consumption (%)	NA	x	NA
Weekly Fruits consumption (%)	NA	x	NA
Feeding frequency (%)	NA	x	NA

x Evaluated; *Mean and frequency variation between baseline and the 12-month follow up; ** frequency variation between the first and the sixth counselling visits; NA – Non applicable.

- The process indicators will comprehend alteration/behavior or practices regarding the data mentioned above (for both educational groups), namely:
- Sustained inadequate behavior:inadequate behavior observed/reported either at the first and the last counselling visit;Sustained adequate behavior: adequate behavior observed/reported either at the first and the last counselling visit;Changed inadequate behavior: inadequate behavior observed/reported at the first counselling visit that changed to adequate behavior at the last visit;Changed adequate behavior: adequate behavior observed/reported at the first counselling visit that changed to inadequate behavior at the last visit;

Statistical analysis

SPSS software (version 25.0, International Business Machines Corporation, Pittsburgh, PA) was used for statistical analyses. Analysis are described for each objective.

Factors associated with the occurrence of Iron Deficiency Anemia (IDA) and Iron-unrelated Anemia (non-IDA);

Multinomial models were fitted, each with a single independent variable and taking children without anemia as the reference category of the dependent variable (vs. IDA and non-IDA anemia). Variables that in those models were significantly associated with any type of anemia, considering a significance level of 10% ($p < 0.10$), were then included as independent variables in a multivariate multinomial model. For those models, the manual stepwise method was used to retain only the variables with an association with anemia, at a significance level of 5% ($p < 0.05$) in the final model. Models considering

all children and stratified by age groups (children under 6 months, between 6 and 23 months and between 24 and 36 months) were fitted. Nagelkerke R square was used to evaluate the goodness of fit of the models.

Impact of zinc deficiency and overload in the occurrence of anemia

Crude multinomial models were fitted, each with each independent variable and taking children with normal zinc serum levels as the reference category for the dependent variable (vs. Low zinc levels and high zinc levels). Logistic regression models were used to estimate the individual effects of independent variables and mediating (intermediate) variables in the occurrence of anemia. Mediation was considered to exist if: 1) the independent variable influenced significantly the mediating variable, 2) the independent variable influenced significantly the dependent variable and, 3) the addition of the mediating variable to the model altered significantly the effect of the independent variable in the dependent one [149, 150]. In addition to this method, Sobel test was used to determine the significance of the mediation effect, as proposed by Baron & Kenny (1986). Interaction is reported to occur when the effect of an explicative variable on the occurrence of anemia is different across the strata of another explicative variable [151, 152]. Here, an interaction was considered to exist if the combined effect of both variables were larger or smaller than the individual effects of A and B in the same model and statistical significance ($p = <0.05$) was observed.

Effectivity of an educational intervention in WASH/Malaria in reducing nutritional anemia, when compared with an educational intervention in nutrition.

Prevalence was calculated as the frequencies of the outcome over the total number of samples with valid results and prevalence reduction (PR) was calculated as the difference between the proportion of prevalence at the baseline *vs* 12-month after the beginning of the study. To investigate the differences in the baseline characteristics, between the children completing the study and the ones dropping out after 12-months, we used Chi-square Test for the categoric variables and T Student for the continuous variables. Logistic regression models were used to analyze interactions between the study arms and variables (using dropouts as dependent variables) and determine if losses were differentiated between arms. Thus, the existence of differences in the baseline characteristics between study arms (for children completing the study) was investigated using ANOVA and Chi-square, respectively to continuous and categoric variables.

Students´ t and McNemar tests were used to determine longitudinal variations on the primary and secondary outcomes between baseline and the end of the study (respectively at the pre and post-interventional moments) within each arm. To determine the differences in the crude variations between the three study arms, the difference-in-difference technique was performed using Fit Generalized estimating models. In summary, linear regression models were used to estimate the mean hemoglobin

level and weight variations and logistic regression models were used to estimate the variation on the prevalence of anemia and other secondary binary outcomes. In each model, independent variables were time (0=T0 and 1=T1), the group, and group*time interaction. Outcomes presenting significant longitudinal variations within each arm, or significant crude differences when comparing study arms, were selected to be further inspected for significant differences in adjusted models. Adjustment was conducted by including secondary outcomes whose prevalence was found to be differentially distributed among the three arms at the baseline (considered as potentially confounding variables) and also age (found to influence the occurrence of anemia in previous studies). The interaction term (represented by DDI) indicates whether there were statistically significant differences in the adjusted variation of T0 to T1 between groups, as previously described by Mahfuz *et al* 2016 [153]. The control group was used as the reference group when determining the effectiveness of the therapeutic-plus-educational intervention, comparatively to an exclusive therapeutic (test-and-treat) intervention, while the nutrition group was used as the reference group when determining the effectiveness of the WASH/Malaria educational intervention (further accounting for the number of interviews conducted in both educational interventions). The quantification of the crude percent of enhancement in the hemoglobin level and in the prevalence of anemia and associated factors was calculated as described by Mahfuz *et al* 2016 [153]. Process indicators were collected at every counselling visits (from a total of six visits) conducted in nutrition and WASH/Malaria arms. Changes in the proportion of adequate behaviour or practices were calculated between the first and the sixth visits, using McNemar tests. Adequate household behavior/practices in the WASH/Malaria arm included observed bednet in the children's bedroom, clean latrine, latrine with water to wash hands (either with current or still water mechanisms), clean backyard environment, namely without garbage, loose animals, still waters and caretaker with clean nails. In the nutrition group, adequate behavior/practices included the reported weekly consumption of cereals, seeds, milk and milk derivatives, food of animal origin, eggs, legumes, vegetables, other fruits and the minimum feeding frequency. "Sustained behavior/practice" was considered to be a behavior or practice observed in both first and sixth visits and "Changed behavior" was considered to be an altered behavior or practice between the first and sixth visits.

Drug therapy at the evaluation moments and follow up waves

Children diagnosed with *Plasmodium falciparum* malaria, urogenital schistosomiasis and/or intestinal parasites, have received treatment, respectively, Artemether/Lumefantrine (20/120, plus paracetamol (15mg/kg/dose for temperatures above 38ºC)), Albendazol (400mg/single dose for *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms) and Praziquantel (40mg/kg for *Schistosoma haematobium* and *Schistosoma mansoni* (splited into two intakes, 6 hours apart) and 25mg/kg for *Hymenolepis nana*), according to the national therapeutic guidelines and specialized bibliography [154-157].

The first dosage of antimalarial was given to the *Plasmodium falciparum* infected children on the evaluation site (and the remaining was given to caretakers to complete the treatment at home, unless signs of severe malaria were observed) and all albendazole treatments (except for children aged younger than one) was administered in the household by a nurse. The remaining infected children were treated at specific consultations with CISA´s paediatrician at the health centres. Furthermore, any children diagnosed with sickle cell disease were referred to the Anemia Patient Follow-up Consultation, held at the Bengo General Hospital. Drugs were provided by CISA through the entire duration of the study.

Ethical considerations and participants' consent

After the explanation of the study, field technicians have provided an informative brochure to the caretakers. Thereafter, they were asked to sign an informed consent, to formalize their acceptance and commitment to participating in this study. At the end of the collection, the data was computerized being accessible only to the principal investigator and the research study coordinators. Treatment and referral protocols were created aiming at treating all children testing positive for malaria, urogenital schistosomiasis and intestinal parasites, at any evaluation moment. The children identified as suffering from severe illness were referred to the emergency pediatric unit at Bengo General Hospital and children with sickle cell disease were scheduled for a specific consultation with a CISA pediatrician in order to receive appropriate treatment and parental counselling. This study was approved by the scientific commission from the ISPUP-FMUP and by the Ethics committee of the Ministry of Health of the Republic of Angola. This study is registered with the title: Efficacy of community educational interventions in nutrition and WASH/Malaria in reducing anemia in under-5 children, in the municipality of Dande – Angola, at

www.isrctn.com (https://doi.org/10.1186/ISRCTN18101157), with the trial identifier number ISRCTN: 18101157. The date of primary registration is 11/04/2016.

General results

The results from this thesis aim at answering to 5 specific objectives: to describe the dynamics of anemia in Angolan and African children population (paper I); to measure the prevalence of anemia and its etiologies (paper II); to describe the main factors separately associated with the occurrence of Iron Deficiency Anemia (IDA) and Iron-unrelated Anemia (non-IDA) (paper II); to study the impact of zinc deficiency and overload in the occurrence of anemia (paper III); and to determine the effectiveness of an educational intervention in Water, Sanitation, Hygiene and Malaria in reducing nutritional anemia, when compared with an educational intervention in Nutrition (paper V). The results from those papers are summarized within this section.

Dynamics of anemia in Angolan and African children population (paper I)

In Angola, the data on what is causing anemia is almost inexistent, as also the available data regarding preventive and control strategies (specifically targeting anemia) implemented within national territory. The Pubmed search resulted in 54 papers, from which only 6 were used for data extraction, while the Google search resulted in 12 pdf files, from which only 3 files were used.

In those studies, it was reported that in 1992 there was a high proportion (near 39%) of children aging between 3 months and 13 years with hemoglobin below 6.8mmol/l, occurring mainly in children from low social and economic state [15]. Later, reports from the WHO, mentioned that the prevalence for Angolan preschool children between 1998 and 1999 was 29.7%, representative of a moderate public health problem, but between 2015 and 2016, the national multiple indicators survey, reported even more alarming prevalence´s (65%) [16, 17]. In that age group, the prevalence was reported to be higher in 6-to-11 months children (83% in 6-8 months and 82% in 9-11 months children) and to have higher severity in 12-17 months children with 9% of moderate-to-severe anemia [17, 18]. Additionally, geographic heterogeneity was documented, with the prevalence ranging from 50% in Lunda Sul, 77% in Cuando Cubango and 57% in Bengo province [18-20].

Regarding the factors associated with anemia in Angola, in 2011, the strong relationship between anemia and malaria was evidenced nationally by the Malaria Indicators Survey conducted in the country [28]. They suggested that malaria-infected children were at a higher risk of anemia, and that areas with the highest prevalence of malaria had higher proportion of severe anemia. Furthermore, associations between the occurrence of anemia and lower social strata was reinforced [28]. Comprehensive studies, conducted in Bengo in 2010, confirmed the association with malaria and added that, along with schistosomiasis they were responsible for 16% and 10% of anemia cases (respectively). Ascariasis was associated with undernutrition, in turn responsible for 13% of anemia cases [19, 20].

Lemos *et al*, tested a therapeutic intervention in children (with 2-15 years) from a highly *S. haematobium* endemic setting of the Bengo province, between 2012-2013 [21]. In sum, authors reported low effectiveness in reducing infections when a mass administration of praziquantel was conjugated with albendazole and an antimalarial test-and-treat approach to malaria-infected children, describing that prevalence reductions one month after treatment were relevant for urogenital schistosomiasis and intestinal parasites, but that 6 months later the prevalences were similar to the ones at the baseline [21]. Additionally, the variation in the prevalence of *P. falciparum* was postulated to have been biased by the seasonality of malaria (which increased one month after treatment but decreased six months after), while the very high prevalence of anemia was observed to have non-

significant reduction [21]. Those results reiterate the high prevalence of infections and anemia in Angola and suggest that the beneficial effect of therapeutic interventions may not be sustained in our setting, possibly due to heavy contaminated environments. The second study reported that anemia was reduced from 54.1% to 13.4% in malaria-infected children treated and from 53.1% to 15.9% in children treated with Artesunate-Amodiaquine, and followed by 28 days (within an efficacy clinical trial) [102].

Prevalence of anemia and its etiologies (paper II)

At the baseline, approximately one half of the children were aged between 6 and 23 months: 517/943 (54.9%) and a similar proportion of boys (50.6%, 479/946) and girls (49.4%, 467/946) was observed. From those with evaluable data, 9.9%, 26.7% and 20.3% were wasted, stunted or underweight, 44.4% were anemic (46.0% of which with IDA), 38.1% had iron deficiency, 8.4% had zinc deficiency and 20.8% had zinc overload. Regarding the feeding practices, 49.3% of the under 6 months children were reported to have been exclusively breastfeed in the previous 24 hours, 52.5% of children older than 5 months were both continued breastfed and complementary fed, only 22.7%, 75.8% and 35.3% of children in that age group achieved the Minimum Dietary Diversity or consumed heme-iron and non-heme iron rich foods, respectively.

In terms of infections and infection preventive practices, 50.9% of the studied children had CRP levels consistent with inflammation (3.3% with concomitant malaria infection). Furthermore, the prevalence of *P. falciparum*, *A. lumbricoides*, *G. lamblia* and *S. haematobium* was 5.2%, 3.8%, 7.5% and 6.3%, and 15.2%, respectively. However, despite being less prevalent, *T. trichiura* (0.5%, 4/787), *E. histolytica* (0.3%, 2/787), *S. mansoni* (0.1%, 1/787), *H. nana* (0.8%, 6/787) and *S. stercoralis* (0.5%, 4/787) were also observed. Diarrhoea in the 2 weeks prior to evaluation was reported in 41.2% of the children. Furthermore, 42.8% (391/913) of the children had slept under the bed net in the previous night, 73.5% (685/932) of the caregivers reported to treat the drinking water, which was obtained mainly from natural sources (50.4%, 467/927), the bathing water was also reported to be mainly obtained from untreated sources (63.1%, 502/796) and despite that 74.4% (694/933) of the caregivers reported to have latrine, 35.0% (327/935) reported to deposit the stool in open sky when outside, and 70.5% (589/835) of children were observed to be wearing shoes at the baseline evaluation.

Genetically, 42.5% (195/459) of the females had at least one G6PD polymorphism (B/A- (29.6%), A/A- (8.3%) and A-/A- (4.6%)), while a similar prevalence occurred in males,43.4% (208/479), and 23.9% (203/848) were found to have the sickle cell trait and 1.9% (16/848) were homozygous for sick cell anemia.

Main factors associated with the occurrence of Iron Deficiency Anemia (IDA) and Iron-unrelated Anemia (non-IDA) (paper II)

Overall, adjusted multivariate regression models associated IDA with age, gender and inflammation and non-IDA with age, zinc deficiency and overload, *P. falciparum*, sickle cell trait/anemia. Among 6-to-23-month-old children IDA was associated with continued breastfeeding and among 24-to-36-month-old children IDA was associated with stunting. Furthermore, zinc deficiency was associated with non-IDA among both age groups children and inflammation was associated with IDA and non-IDA in either 6-to-23 and 24-to-36 months old children (see table 3).

Table 3 – Multinomial multivariate regression models for IDA and non-IDA.

Independent variables		**Non anemic**	**IDA** OR (IC95%)	***p***	**Non-IDA** OR (IC95%)	***p***
Total population (1)						
Age	< 6 months	1	Ref		Ref	
	6 - 23 months		7.4 (2.87, 19.11)	<0.001	0.7 (0.43, 1.15)	0.166
	24 - 36 months		2.0 (0.73, 5.53)	0.180	0.5 (0.27, 0.80)	0.006
Children´s gender	Female	1	Ref		Ref	
	Male		2.0 (1.32, 2.91)	0.001	1.3 (0.87, 1.81)	0.216
Zinc	Normal	1	Ref		Ref	
	Deficiency		1.6 (0.67, 3.61)	0.306	3.2 (1.64, 6.25)	0.001
	Overload		0.8 (0.47, 1.26)	0.300	0.6 (0.36, 0.96)	0.033
P. falciparum	No	1	Ref		Ref	
	Yes		1.3 (0.26, 6.81)	0.733	3.1 (1.05, 9.42)	0.041
Inflammation	No	1	Ref		Ref	
	Malarial Inflammation		3.8 (0.56, 25.70)	0.174	1.8 (0.44, 7.36)	0.415
	Non-malarial Inflammation		2.4 (1.62, 3.65)	<0.001	1.3 (0.90, 1.94)	0.157
Sickle cell (*HBB* genotype)	AA	1	Ref		Ref	
	AS		1.00 (0.59, 1.55)	0.853	1.6 (1.03, 2.35)	0.035
	SS		1.2 (0.10, 13.54)	0.904	16.6 (3.56, 77.04)	<0.001
Children under 6 month (2)						
Age	Continuous variable	1	-	-	1.3 (1.02, 1.57)	0.031
Zinc	Normal	1	-	-	Ref	
	Deficiency		-	-	1.1 (0.20, 5.85)	0.927
	Overload		-	-	0.3 (0.12, 0.73)	0.008
Children 6 to 23 months (3)						
Gender	Female	1				
	Male		2.1 (1.34, 3.27)	0.001	1.3 (0.78, 2.10)	0.321
Continued breastfeeding	No	1				
	Yes		1.9 (1.11, 3.13)	0.019	1.6 (0.92, 2.90)	0.095
Zinc	Normal	1				
	Deficiency	1	1.4 (0.42, 4.48)	0.604	4.4 (1.55, 12.28)	0.005
	Overload		0.8 (0.44, 1.30)	0.307	0.7 (0.35, 1.27)	0.221
Inflammation	No	1				
	Malarial Inflammation		2.3 (0.43, 11.95)	0.331	9.1 (2.34, 35.71)	0.001
	Non-malarial Inflammation		2.2 (1.42, 3.47)	<0.001	1.5 (0.90, 2.48)	0.119
Children 24 to 36 months (4)						
Age	Continuous variable	1	0.9 (0.77, 0.98)	0.020	1.0 (0.955, 1.13)	0.408

Zinc	Normal	1				
	Deficiency		1.4 (0.38, 5.28)	0.609	3.6 (1.41, 9.09)	0.007
	Overload		1.0 (0.26, 4.14)	0.960	0.7 (0.23, 2.35)	0.605
Stunting	Normal	1				
	Moderate to severe		2.6 (1.09, 6.20)	0.031	1.2 (0.60, 2.23)	0.675
Inflammation	No					
	Malarial Inflammation	1	18.2 (3.55, 92.76)	<0.001	2.3 (0.54, 9.94)	0.262
	Non-malarial Inflammation		4.0 (1.45, 11.01)	0.007	0.4 (0.21, 0.90)	0.024

Only variables with a significance level of 10% ($p < 0.10$) were included as independent variables in a multivariate multinomial model. Model adjustment (1): Pearson: $\chi2(170) = 162.9$, $p = 0.638$; Deviance: $\chi2(170) = 169.9$, $p = 0.488$; R2 Nagelkerke = 0.208. Model adjustment (2): Pearson: $\chi2(22) = 43.1$, $p = 0.500$; Deviance: $\chi2(22) = 48.6$, $p = 0.100$; R2 Nagelkerke = 13.5%. Model adjustment (3): Pearson: $\chi2(40) = 27.3$, $p = 0.938$; Deviance: $\chi2(40) = 31.0$, $p = 0.845$; R2 Nagelkerke = 13.0%. Model adjustment (4): Pearson: $\chi2(176) = 170.5$, $p = 0.604$; Deviance: $\chi2(176) = 167.0$, $p = 0.675$; R2 Nagelkerke = 20.3%

Impact of zinc deficiency and overload in the occurrence of anemia (paper III)

Children with high zinc levels were significantly less likely of being infected with *P. falciparum*, and children with zinc deficiency had 1.9 more chances of having non-malarial inflammation

Regarding the investigation of the possible plausible particularities in the relation between total anemia and zinc deficiency, namely the participation of iron deficiency, inflammation or infections in their causal pathway, we only found that zinc deficiency significantly interacts (OR: 13.26, p= 0,022) with the presence of at least one intestinal/urogenital parasite in the causal pathway to anemia and that the interaction appeared to be stronger among children with IDA (OR: 46.66, p= 0.003). Also, no mediator or interactive effects among zinc deficiency and iron deficiency, nor significant evidences that inflammation could be mediating or interacting with zinc deficiency to cause anemia and mediator effect between zinc deficiency and infections in the causal pathway to anemia was found. Table 4 - Results of the main effects investigated, occurring between zinc and the studied variables in the pathway to anemia.

Mediation		**Interaction**	
Effect tested: OR (95% CI), p-value		**Effect tested: OR (95% CI), p-value**	
Model 1: Iron Deficiency, Zinc deficiency and anemia (N = 673)			
Direct effect (ZD and anemia)	1.73 (1.26-2.36), p=0.001	Direct effect (Iron deficiency and anemia)	2.79, 1.42- 5.50, p=0.003
Mediated by ZD	1.77 (1.29-2.44), p< 0.001*	Interacted (ZD*ID)	0.39, 0.12-1.31, p=0.127
Model 2: Zinc deficiency, inflammation and anemia (N = 686)			
Direct effect (ZD and anemia)	2.03 (1.16-3.54), p = 0.013	Direct effect (Inflammation and anemia)	1.38 (0.60, 3.16), p=0.444
Mediated by inflammation	1.93 (1.10-3.38), p = 0.022*	Interacted (ZD*inflammation)	1.86 (0.5- 5.87), p=0.290
Model 3: Zinc deficiency, parasites and anemia ZD (N = 595)			
Direct effect (ZD and anemia)	2.30 (1.26-4.22), p=0.007	Direct effect	1.57 (0.81-3.02), p=0.182
Mediated by parasites	2.39 (1.30-4.40), p=0.005*	Interacted (ZD*parasites)	13.26 (1.46-120.72), p=0.02

* p-value (Sobel Test) > 0.05. ** p-value (Sobel Test) < 0.05. ZD – Zinc deficiency, ID – Iron Deficiency, Parasites: *S. haematobium*, *A. lumbricoides*, *T. trichiura*, *G. lamblia*, *H. nana*, *E. hystolitica* and *S. mansoni*.

Effectiveness of an educational intervention in Water, Sanitation, Hygiene and Malaria in reducing nutritional anemia, when compared with an educational intervention in Nutrition (paper V).

Our results from crude difference-in-difference models showed no statistically significant differences between the 3 interventions, regarding the reduction of anemia and increasing of Hb (see table 4). Nevertheless, differentiated variations between groups were found to occur in the prevalence of Iron Deficiency, Zinc deficiency and *P. falciparum* malaria (Wald chi-square p-value = 0.04, 0.04 and <0.001, respectively). Those differences occurring specially between the control and the nutrition arms (Wald chi-square p-value=0.077, p-value=0.015 and p-value=0.001, respectively).

When the regression models were adjusted, for the variables presenting differentiated prevalence profile at the baseline (and taking the control arm as the reference), we found that the nutrition group presented higher percentual enhancement in the prevalence of iron deficiency and lower enhancement of zinc deficiency and *P. falciparum* malaria prevalence. On the other hand, the WASH/Malaria group presented lower enhancement of zinc deficiency prevalence.

Furthermore, when we compared the adjusted WASH/Malaria educational effect to the Nutrition educational effect, we observed that children in the WASH/Malaria group had 22.8% higher prevalence of anemia than the nutrition group. There were also higher prevalence of *P. falciparum* malaria and reported feeding frequency. We found no statistically significant associations between the variations in the outcomes and the number of domiciliary visits provided.

Table 5 – Effectiveness of interventions in reducing anemia and their associated factors.

Type of models	**Crude models**	**Adjusted model 1**				**Adjusted model 2**		
Comparations	**Control vs. WASH/Malaria vs. Nutrition**	**Nutrition vs. Control**		**WASH/Malaria vs. Control**		**WASH/Malaria vs. Nutrition**		**WASH/Malaria vs. Nutrition Group with Interview score effect**
Statistic evaluators	Wald chi-square p-valu	% enhance.	DDI (p-value)	% enhance.	DDI (p-value)	% enhance.	DDI (p-value)	DDI (p-value)
Health primary outcome								
Hb (Mean)	0.29	2.0	+0.151 (0.468)	0.7	0.023 (0.912)	-1.3	-0.160 (0.371)	0.023 (0.403)
Anemia (% yes)	0.22	-17.2	-0.424 (0.273)	0.3	0.263 (0.498)	22.8	**+0.641 (0.050)**	-0.048 (0.332)
Health secondary outcomes								
IDA (% yes)	0.11	50.5	+0.468 (0.351)	24.2	+0.276 (0.606)	-27.3	-0.228 (0.594)	0.017 (0.776)
Iron deficiency (% yes)	**0.04**	71.6	**+0.866 (0.032)**	-5.3	+0.278 (0.498)	-47.5	-0.664 (0.144)	0.088 (0.258)
Zinc deficiency (% yes)	**0.04**	-94.9	**-3.336 (0.006)**	-103.7	**-2.126 (0.088)**	169.5	+1.194 (0.117)	0.081 (0.411)
Weight (Mean)	0.88	0.6	+0.258 (0.291)	0.6	+0.281 (0.253)	0.0	-0.029 (0.886)	-0.006 (0.843)
Stunting (% yes)	0.29	23.3	+0.196 (0.610)	40.4	+0.217 (0.575)	11.8	0.043 (0.89)	-0.041 (0.425)
Underweight (% yes)	0.55	-	-	-	-	-	-	-
Wasting (% yes)	0.44	-	-	-	-	-	-	-
P. falciparum (% yes)	**<0.001**	-317.7	**-3.052 (0.001)**	-669.0	-0.637 (0.433)	569.1	**2.495 (0.013)**	-107 (0.351)
At least one intestinal/urogenital parasite (% yes)	0.79	-129.1	-0.073 (0.867)	-6.9	-0.316 (0.445)	49.8	0.244 (0.510)	-0.061 (0.301)
Inflammation (% yes)	0.35	-	-	-	-	-	-	-
Behavior Secondary outcome								
Green leaf intake (% yes)	0.66	2,3	+0.261 (0.516)	15,9	+0.368 (0.385)	11.7	+0.103 (0.763)	0.009 (0.874)
Meat cons. (% yes)	0.72	-12,0	-0.230 (0.587)	-10,6	-0.262 (0.516)	-1.1	-0.058 (0.877)	0.048 (0.358)
Feeding frequency (Mean)		-15,4	-0.345 (0.164)	-15,8	-0.073 (0.769)	2.6	**+0.416 (0.049)**	0.006 (0.864)
MDD (% yes)	0.73	-	-	-	-	-	-	-

Hb – Hemoglobin, IDA – Iron deficiency anemia. *difference-in-difference Wald chi-square, # Moderate-to-severe. **Models 1** - Yit = β0 + β1 Time + β2 Group + δ (Time × Group) + β3 Gender + β4 Age + β5 Zinc Deficiency + β6 Feeding frequency + β7 Green leaf intake + β8 At least one intestinal/urogenital parasite +ε. **Model 2** (nutrition-WASH/Malaria) and 2 (nutrition-WASH/Malaria with interview scores) - Yit = β0 + β1 Time + β2 Group + δ (Time × Group) + β3 interview score + β4 Gender + β5 Age + β6 Zinc Deficiency + β7 Feeding frequency + β8 Green leaf intake + β9 At least one intestinal/urogenital parasite +ε

In the present study, conducted in the Dande municipality (Bengo province of Angola) in 2015, the prevalence of anemia in under-5 children was 44.4%, markedly lower than previously reported for the country and also for the municipality [17, 18, 20]. This fact could suggest a decrease of anemia in under 5 months children within these areas, following the general trend of prevalence reduction reported for some parts of the world between 1990 and 2013. However, it should be noticed that the sampling design of this study does not represent all the municipality population. Prevalence's were higher in children aged between 6-to-23 months (52%) than in under 6 months (36.1%) and children aged between 24-to-36 months (34.9%), corroborating the national estimates reported higher prevalence's for children aged between 6-to-11 months (reaching near 83% in 6-8 months children, but contrasting with Kassebaum *et al* 2014, who report that the highest anemia prevalence occurs between one month and 1 year of age (at the post-neonatal period), followed by age 1 to 4 years [3].

In our study the prevalence of IDA represented almost half of the anemia cases (46%), in accordance with previous reports. In turn, IDA was found to be associated gender, inflammation, and age. This differential occurrence of IDA among gender may be related to reports of males having lower birth iron stores (possibly determined by in utero hormonal influences), and higher rates of iron deficiency than female infants. The differential occurrence of IDA among children with or without inflammation may be related to infection-related factors. Nevertheless, both associations need further investigation. Multivariate regression models were additionally estimated to better understand the differential occurrence of IDA among age strata. Thus, the age-stratified analysis showed that inflammation continued influencing the occurrence of IDA anemia in both 6-to-23 months and 24-to-26 month, while gender influenced mainly 6-to-23 months children, but nothing was concluded regarding under 6 months children due to invalid models caused by numeric problems. Moreover, additional factors influencing the occurrence of IDA among those age strata were highlighted. For instance, the feeding practices were found to be relevant among 6-to-23 months, as being continued breastfeeding increased the susceptible of having IDA, when compared with children that were in exclusive complementary feeding. This is in accordance with others that reporting differential prevalence among exclusively and predominantly breastfed children, and may argue to the fact that the absorption of iron from breast milk may be happening as required to meet the amount required for growth, the introduction of complementary foods may not be increasing iron intake as expected, and or the consumption of high content of inhibitors of iron absorption may be decreasing the iron from both iron sources. Other possibility would be that non-breastfeed children could be having higher Minimum Dietary Adequacy than those who are continued breastfeeding because formula and/or milk could be more commonly given to them in substitution of a proper meal [57].

Also, among 24-to-26-month children stunting was found to increase 2.6 times the probability of having IDA which corroborate the link between iron deficiencies and possibly long periods of nutritional restriction that leads to stunting. The effect of both inadequate feeding practices and prolonged nutritional restriction may have been exacerbated by high iron requirements posted by rapid growth and physiological state.

We also found that non-IDA anemia was similarly associated with age (with older children being less susceptible), and additionally to zinc deficiency and overload, *P. falciparum* infection, sickle cell trait and sickle cell anemia. From those, only children with zinc overload was observed to be less susceptible to non-IDA. Age-stratified analysis showed that zinc overload protect under 6-month children from non-IDA and zinc deficiency increased the risk in either 6-to-23-month and 24-to-36 months children and inflammation was also important in the occurrence of non-IDA in the last 2 age groups. In general, those results suggest different implied contributing factors, namely possible differential practices or behaviours that prevent infections among age strata (could contribute to different infectious profile), the direct effect of infections (in the case of malaria), the expected influence of genetic diseases (in the case of sickle cell and sickle anemia) and nutritional immunity associated with zinc levels (which could be influencing the occurrence of infections and their impact in anemia). For instance, malaria may lead to anemia by causing acute and chronic hemolysis and subsequent suppression of erythropoiesis, possibly being also influenced by deficiencies in vitamin A, E, riboflavin, folate, selenium and zinc deficiencies (besides iron), either having a protective and/or adverse effect in the development of malarial anemia, while children with sickle cell anemia are reported to present low hemoglobin values (due to hemolysis, evidenced after 4–6 months of age). Finally, zinc levels were reported to influence pro-and anti-inflammatory pathways, which can both lead to immune dysfunctions that worsen the responses towards some infections (zinc deficiency) or protecting against other infections (namely with enteric bacteria when zinc overload occurs).

Based on the reported biological plausibility, regarding the participation of zinc levels in nutritional immunity and on the variability of anemia, and the suggestive behavior of our data in favor of that plausibility we conducted additional exploratory analysis to further investigate this issue. We analytically tested if parasites and/or other pathological conditions could be causing inflammation, which could be mediating or interacting with zinc deficiency in the causal pathway to anemia, however, no statistical significance was observed regarding the mentioned effects. We also tested the hypothesize that zinc could be mediating or interacting directly with parasites to cause anemia (without the influence of inflammation), and despite that no evidences of mediation relations were found, having at least one intestinal and/or urogenital parasites was found to interact with zinc deficiency, increasing significantly the odds of IDA (OR: 46.66, p= 0.003). This could be in accordance with reports mentioning that the body also responds to pathogens by decreasing iron availability, further removing

iron from circulation and storing it as ferritin and zin may participate in this process. Additionally, zinc deficiency may be causing non-IDA anemia directly or by other mechanisms than the ones investigated here.

After describing the prevalence of anemia, its main determinants, their associations and some of their particular associative relations, we tested if a test-and-treat approach could reduce infections and their related anemia; if by adding WASH/Malaria education would comprehend higher achievements in reducing infections-related anemia; or if adding a nutrition education would comprehend higher achievements in reducing nutritional anemia, over a 12-month period. Our crude and adjusted difference-in-difference models suggest that implementing two semesterly rounds of an exclusive test-and-treat therapeutic approach have no statistical significative difference in reducing anemia and increasing Hemoglobin, that when combining this approach with 6 monthly domiciliary counselling visits addressing adequate child nutrition or WASH/Malaria practices. Considering the positive results reported by others, and the fact that our process indicators suggest the occurrence of some degree of behavior change in both educational intervention groups, these effectiveness results could suggest that, 1) the therapeutic component of the intervention played in fact the major role and educating had a residual effect, or 2) not all educational actions have been translated into changed behavior and in consequence the needed sequence of beneficial results happened incompletely and/or 3) the educational actions lacked the required intensity (number of monthly visits and duration of each session) and duration of the educational actions (only 12-months) to translate into significant changes at the primary outcomes [102, 158].

Additionally. we have analysed our data by protocol, rather than by intention to treat, to allow combining different types of evidences able to assist the interpretation of our results and to provide a more comprehensive discussion following the assumption mentioned above [159]. Instead of observing reduced prevalence of infections, as occurred in other studies, we observed a significant increase in the prevalence of the studied infections in all study groups (with exception of the decreased prevalence of *P. falciparum* observed in the nutrition group). Those variation were accompanied by high occurrence of new cases and reinfections, suggesting a frequent/repetitive contact of children with highly contaminated environment, in particular with water bodies contaminated with cercaria, frequent exposure to mosquito bites, possible ingestion of eggs and quists of intestinal parasites in contaminated drinking water and/or food and skin exposure to soil contaminated with infectant larvae. This assumption its corroborated by reports of substantial increased prevalence of infections in the first 5 years of age, due to increasing contact/exposure with/to contaminated sources, as well as a more immature and less reactive immunity system.

Indirectly, those infections and excessive loss or competition for hematopoietic nutrients may have influenced the occurrence of nutritional anemia and the effect of the interventions in the primary and

secondary outcomes. Nevertheless, despite the increased prevalence of infections in the control and WASH/Malaria groups, we observed a statistically significant reduction of IDA´s prevalence in both groups (-13.2% in the control group and -8.4% in the WASH/Malaria group). Thus, we postulate that the effect of the therapeutic component of this intervention in the health secondary outcomes (infections) may have been only temporary (due to the high incidence and reinfection rates), but even that partial achievement (which limited the impact of infections), allowed that a normal diet and physiologic processes restored partially the nutritional status (reduced the IDA related with infections). When the WASH/Malaria group was compared to the control using a difference-in-difference technique, only differences in the zinc deficiency prevalence were found (with the WASH/Malaria group having lower prevalence), suggesting that adding the educational component had limited relevance in the variations of the outcomes. However, our process indicators suggest that the educational component of the intervention may have had impacted in only some behavioral secondary outcomes (measured by changes of inadequate to adequate behavior/practice between the first and last educational visits) but not in all of them, possibly because they weren't of enough intensity or duration to allow blocking the transmission of infections from happening.

On the other hand, we postulate that the educational component of the intervention may have had positive impact in some behavioral secondary outcome (but not in all of them), who in turn (and additionally to the temporary beneficial effects of cleared infections) may have positively influenced the nutritional primary and secondary health outcomes studied here. For instance, despite that a significant increase in the prevalence of reported consumption of green leaf (18.2%) was documented between the baseline and the 12-month follow up wave, children were reported to have significantly consumed less meet (-16.9%) and having lower feeding frequency (-0.26) at the end of the study when compared to the baseline. Also, the educational component of this intervention failed at increasing the minimum dietary diversity which, considered the facts explained above, would possibly contribute to the positive effect that the increased green leaf consumption must be having in the Hb or anemia. Nevertheless, we observed either increased Hb mean and decreased total anemia prevalence, despite that increased prevalence of *stunting* was also observed. When compared with the control group using a difference-in-difference technique, the nutrition arm showed significantly higher zinc deficiency and lower *P. falciparum* infections, and when compared with the WASH/Malaria group we observed that this last group presented higher rates of total anemia and malaria. When we included of the number of successfully domiciliary counselling visits received by the caretaker's interviews in the comparison of WASH/Malaria and nutrition groups, no relevant alterations were observed.

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Paper I: Anemia in preschool children from Angola: a review of the evidence

Review Article

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Anemia in preschool children from Angola: a review of the evidence

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Abstract

Angola is one of the southern African countries with the highest prevalence of anemia, and despite the high geographic heterogeneity of its distribution across the country, it was reported to be indicative of a severe public health problem in some areas, mainly in children. Despite the relevance of this condition in the country there is still an important gap regarding scientific evidences and knowledge systematization in the indexed literature, that could be used to inform and optimize national public health policies willing to address it. Furthermore, the changes in anemia epidemiology among African preschool children and the late updates in nutrition-specific and nutrition-sensitive preventive strategies in the continent are of imperative relevance, as they could contribute to design context-specific national approaches to reduce anemia's morbidity and mortality. In this study we intent to perform a systematic review regarding the sparse evidence available on the country regarding the prevalence of anemia, its associated factors, the prevention, and/or control strategies with potential to reduce anemia that were implemented, and to discuss interventions targeting infections and/or nutrition conducted in other African countries.

Keywords: Africa, anemia, Angola, associated factors, preschool children, prevalence

Introduction

The prevalence of anemia in 2011 for 6- to 59-month children from the WHO's African region, was 62%, presenting a substantial geographic variability.[1] For instance, in that year, Tunisia, Libya, Algeria, Morocco, Seychelles, and Rwanda had the lowest prevalence's in Africa (29%–38%), followed by South Africa, Botswana, Djibouti, Mauritius, Swaziland, Egypt, Kenya, Burundi, Lesotho, Namibia, Ethiopia, and Madagascar (41%–50%); Comoros, Angola, Uganda, Somalia, Zambia, Eritrea, Sao Tome and Principe, Sudan, Zimbabwe, Cabo Verde, and Gabon (51%–60%); Tanzania, Cameron, Benin, Congo, Gambia, Malawi, Mozambique, and Democratic Republic of Congo (61%–67%); and the remaining countries of the continent having the highest prevalence's (71%–86%).[1] Temporal variations were also observed among those countries, presenting sex- and age-specific variabilities.[2–5] For instance, the number of countries with prevalence's >50% decreased significantly between 1990 and 2010, decreasing more for males and having negative trends in children younger than 5 years.[6–9]

Consistently, this prevalence variability is also followed by variations in the morbidity associated to this condition. For instance, age-adjusted analysis in studies conducted in 2013, showed that the burden of anemia was more concentrated in western and central sub-Saharan Africa, possibly indicating a greater severity of disease and higher prevalence of its etiologies in those regions.[6] Kassebaum et al,[9] 2014 reported that areas where the prevalence of anemia is high, may also have higher burden of anemia related to infectious and iron-related etiologies. In accordance, the achievements reported for the Eastern sub-Saharan Africa in 2013 may possibly be also attributed to the implementation of programs that have likely reduced the causes of anemia, such as improvements in health, poverty, and living conditions (unrelated to the health system).[6,9] Nevertheless, the combination of improvements leading to those changes still need further clarification. Also, the context-specific etiologic profile of anemia was also suggested to be a crucial factor, influencing anemia's associated mortality.[10–15] For instance, in Kenia, malaria, bacteremia, and chronic diarrhea were reported to be responsible for 12.2%, 8.7%, and 32% of the anemia-related deaths, contrasting with Ghana where nutritional anemia, severe malaria, and sickle cell anemia caused respectively, 52.1%, 21.6%, and 10.8% of those deaths.[11–14] Furthermore, in Zaire, severe anemia caused by iron deficiency was reported to be the second greatest cause of death in children younger than 5 years, and in Gambia, the seasonal variations of malaria and anemia were similar, with the peak mortality in young children occurring when both were most prevalent.[11,15–17]

In Angola, the southern African country with the highest prevalence rate in 1990, Kassebaum et al[6,9] 2016 reported a similar tendency of decreasing prevalence (from 50%–60% to 40%–50% for all ages). However, the availability of holistic and public health-relevant data concerning the anemia associated factors, that could in turn be used to design adequate and

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context-specific interventions, is inexistent. In this study we intent to (1) perform a systematic review regarding the sparse evidence available on the prevalence and factors associated with the occurrence of anemia in the country and regarding prevention and/or control strategies that were implemented in national territory with potential to reduce anemia and (2) to discusses interventions targeting infections and/or nutrition conducted in other African countries.

Methods

The MESH terms were defined according to the research question. In general, they were introduced in the PubMed to screen for scientific papers, whereas Google search was used to screen for relevant reports. After introducing the MESH terms in the databases and research engines, the abstracts returned from the search were inspected for eligibility. Furthermore, studies were considered eligible if they reported in the abstract the prevalence of anemia, its related factors, and associations or interventions conducted. Studies conducted in animals, population older than 4 years, outside of Africa, regarding hereditary or acquired hemolytic or aplastic anemia, nontarget infections (HIV, etc) or in other language than English were excluded. After selecting the review papers for complete reading, the data relevant to each research question was extracted and resumed for integration in the respective section of this article. Back and forward cross references were conducted whenever found relevant.

Research question: prevalence of anemia and its associated factors in children younger than 5 years in Angola

Pubmed search. To investigate all studies related to anemia conducted in Angola we used the MESH "("anaemia"[All Fields] OR "anemia"[MeSH Terms] OR "anemia"[All Fields]) AND ("Angola"[MeSH Terms] OR "Angola"[All Fields])", from this search resulted 54 papers. After considering the exclusion criteria, 46 papers were excluded: 35 for comprehending unrelated diseases, condition, or scope (mainly hemolytic hereditary anemia), 2 studies for unsuitable design (study protocol or laboratory methodological research), 5 studies due to noninclusion of under 5 years old, 3 for unavailable abstract and 1 conducted outside Angola. The full texts of the remainder 9 papers were then critically reviewed. From those, 2 policy papers and 1 reporting results from another paper already reviewed were excluded. The remaining papers were resumed in a table.

Google search. For this search we used the keywords: "Anemia em Angola" plus "Anemia in Angola," to include both results in Portuguese and English. Furthermore, only pdf files resulting from the search were inspected for eligibility. This search resulted in 12 pdf files, 11 in Portuguese and 1 in English. After full text inspection, 9 documents were excluded because their main scope was unrelated to anemia or associated etiologies, and data regarding the prevalence of anemia in preschool children was extracted from the remaining 3 files.

Results

Review of evidence—anemia in Angola

There is lack of evidence in Angola regarding the prevalence of anemia and the determinants that influence its occurrence. In addition, concerning is the fact that (as far as we know) there are no published studies specifically targeting the prevention or control of anemia. In this study, the data on what is causing anemia are almost inexistent, as also the available data on preventive and control strategies implemented within national territory. The results of studies that include prevalence of anemia and/or their etiologies and studies of interventions impacting the anemia's occurrence are presented here.

Prevalence and associated factors in Angola. It was reported that in 1992 there was a high proportion (approximately 39%) of children aging between 3 months and 13 years with hemoglobin <6.8 mmol/L, occurring mainly in children from low social and economic state (in which the anemia frequency was in turn associated with physical development).[18] Later, reports from the WHO mentioned that the prevalence for Angolan preschool children between 1998 and 1999 was 29.7%, representative of a moderate public health problem.[19] In 2011, the strong relationship between anemia and malaria was evidenced nationally by the Malaria Indicators Survey conducted in the country, suggesting that malaria-infected children were at a higher risk of anemia, and that areas with the highest prevalence of malaria had higher proportion of severe anemia (4% of the children in the hyperendemic region had hemoglobin lower than 8.0g/dL, whereas 2% of the children living in the hipoendemic region of Luanda had it). Furthermore, associations between the occurrence of anemia and lower social strata were reinforced.[20] Between 2015 and 2016, the national multiple indicators survey, reported alarming prevalence in children younger than 5 years (65%).[21] In that age group, the prevalence was reported to be higher in 6 to 11 months children (83% in 6–8 months and 82% in 9–11 months children) and to have higher severity in 12–17 months children with 9% of moderate-to-severe anemia.[21] In addition, geographic heterogeneity was documented, with the prevalence ranging from 50% in Lunda Sul and 77% in Cuando Cubango.

Regionally, comprehensive studies conducted within the Dande municipality of the Bengo's province in 2010 reported that the prevalence of anemia in children younger than 5 years was 57% and that malaria and schistosomiasis were responsible for 16% and 10% of anemia cases, whereas ascariasis was associated with undernutrition (in turn responsible for 13% of anemia cases).[22,23] In that area, the distribution of anemia was found to be heterogeneous and to be influenced by the distribution of malaria. Between 2012 and 2013, Lemos et al[24] tested a therapeutic intervention in children (with 2–15 years) from a highly *Schistosoma haematobium* endemic setting of that province in which the prevalence of anemia at the baseline was very high (76%). In the studied children, 76% had *S haematobium*, 31% had at least 1 soil-transmitted helminth, 10% had *Hymenolepis nana*, and 6.7% had *Plasmodium falciparum*. The study also reports that, among the children followed, all anemia cases were infected with *S haematobium*.[24]

Interventions with potential two reduce anemia implemented in Angola. Using the described methodology, we have documented only to interventional studies. Lemos *et al*,[24] tested a therapeutic intervention in children (with 2–15 years) from a highly *S haematobium* endemic setting of that province in the Bengo province, between 2012 and 2013. In sum, authors reported low effectiveness in reducing infections when a mass administration of praziquantel was conjugated with albendazole and an antimalarial test-and-treat approach to malaria-infected children, describing that prevalence reductions 1 month after

treatment were relevant for urogenital schistosomiasis and intestinal parasites, but that 6 months later the prevalences were similar to the ones at the baseline.[24] In addition, the variation in the prevalence of *P falciparum* was postulated to have been biased by the seasonality of malaria (which increased 1 month after treatment but decreased 6 months after), whereas the very high prevalence of anemia was observed to have nonsignificant reduction.[24] Those results reiterate the high prevalence of infections and anemia in Angola and suggest that the beneficial effect of exclusively therapeutic interventions may not be sustained in our setting, possibly due to heavy contaminated environments. On the contrary, the effectiveness of nutrition-specific interventions needs also to be further investigated. The second study reported that anemia was reduced from 54.1% to 13.4% in malaria-infected children treated and from 53.1% to 15.9% in children treated with Artesunate-Amodiaquine, and followed by 28 days.[25]

Discussion

Main strategies to reduce anemia

Resulting from the review of the evidence available in Angola that was mentioned above, the prevalence suggests the occurrence of anemia in children younger than 5 years as a severe public health problem, in turn associated with either proximal or distal determinants. The main immediate determinants reported are infections, mainly malaria and schistosomiasis. Furthermore, malnutrition has been regionally associated, but data available are very scarce. In fact, designing strategies to reduce anemia requires previous baseline characterization that includes determining the context-specific profile, composed by documentation of anemia's frequency, spatial distribution, risk groups affected, and the contribution of its determinants. This review was unable to provide the necessary data due to scarce and/or publicly unavailable results.

Nevertheless, based on general knowledge, nutrition-specific approach, targeting mainly the immediate determinants of anemia, a nutrition-sensitive approach, targeting fundamental/basic, underlying and intermediate determinants, or a combination of both should be considered.[26] Although defining the immediate determinants (nutritional, infectious, and/or genetic) would allow designing short-term control strategies, and determining the relevance of fundamental/basic, underlying, and intermediate determinants would allow designing medium-to-long term prevention strategies, a combination of both would comprehend control and preventive dimensions and could tackle anemia more comprehensively.[8,16,27] For the purpose of guiding future strategies targeting nutritional anemia in Angola, the rational for some nutrition-specific and nutrition-sensitive approaches are resumed bellow as also the results from some interventional African studies.

Nutrition-specific approach: food-based interventions. Food-based interventions can aim at (1) increasing the production, availability, and access to micronutrient rich foods, (2) increasing the consumption of micronutrient-rich foods (improving either the overall quantity or quality of multiple erythropoietic micronutrient), (3) increasing the bioavailability of micronutrients in the diet (by increasing the consumption of enhancers and decreasing the consumption of inhibitors of nonheme iron absorption), or (4) combining some of those aspects.[8,28–30] Their overall goal is to empower individuals to improve the quality of their own diets and are reported as being sustainable for preventing anemia (mainly Iron Deficiency Anemia) or related micronutrient deficiencies.[8,27,31,32] These approaches can be delivered through education, communication, social marketing, and behavior changing programs to target specific groups, and in young children they are frequently oriented to improve age-adequate breastfeeding, dietary diversity (frequently accounting for increasing micronutrient bioavailability), and feeding frequency.[7,16,31,33–43] Considering its relevance, the diet diversification and micronutrient bioavailability strategies are briefly discussed below.

Diets in rural areas from developing countries were reported to be based mainly in cereals, starchy roots, and/or tubers and legumes, having normally less frequent consumption of dairy products and flesh foods (which are rich sources of readily available heme-iron, zinc, and vitamin A as retinol, probably associated to disadvantaged dynamics of anemia-related determinants mentioned above.[44,45] Plant-based diets are reported to have high content in phytic acid, dietary fibers, and polyphenol, components that may inhibit the absorption of important trace minerals (mainly nonheme iron and zinc), and unsurprisingly, that pattern was reported not to provide the daily needs of calcium, iron, and zinc.[28,29,46–49] In addition, they frequently have low fat content, which further limit the absorption of lipid-soluble vitamins. When considering dietary diversification to tackle micronutrient deficiency in those settings, it should be considered that the simultaneous consumption of nonheme iron-rich foods, and enhancers of iron absorption (citric, malic, or ascorbic acid) or heme iron-rich foods (such as meat, poultry, or fish) may improve iron absorption by limiting the effect of iron inhibitors.[50] Furthermore, it should also be taken into account that the consumption of legumes, green leafy vegetables, whole grains, and fruits/fruit juices may potentially increase folate levels naturally, the consumption of green leafy vegetables, orange and/or yellow fruits, vegetables, dairy products, eggs, and fish may increase vitamin A levels, and animal-source foods (such as shellfish, meats, fish, poultry) and dairy products may increase the levels of vitamin B12.[16] Furthermore, some methods of food processing (soaking, fermentation, germination, or thermal or mechanical processing) may also alter the bioavailability and absorption of micronutrients.[16,28]

Nutrition-sensitive approach—preventing and reducing infection-related anemia. Infections can cause anemia indirectly by causing malabsorption of nutrients or anorexia, but also directly through hemoglobin loss (by blood loss in urine and stool, hemolysis) or impaired red blood cell production (through inflammatory response).[51–53] Hookworms (*Necator americanus* and *Ancylostoma duodenale*) and schistosomes (particularly *S haematobium*) were reported to be the largest direct contributions to anemia through blood loss.[1,5,54–56] For instance, published report estimates that one half of moderate-to-severe cases of anemia in children may occur due to hookworm infection.[7,16,57] Nevertheless, the intensity of infection, and coinfection with multiple parasites determine the severity of blood loss.[16,57] In addition, schistosomiasis may also contribute to anemia through splenic sequestration of erythrocytes, increased hemolysis, or inflammation due to chronic disease.[16] Besides leading to a less severe blood loss, *Ascaris lumbricoides* infection also influences the iron status through gastric and intestinal ulceration and others such as *Trichuris trichiura* or *Giardia lamblia* are associated with malabsorption of micronutrients. Malaria associates with severe anemia by causing acute

and chronic hemolysis, subsequent suppression of erythropoiesis, and possibly secondary folate deficiency.[8,16,58,59] Because enteric nematodes, cestodes, trematodes, and protozoans are often co-endemic, polyparasitism has been suggested to have interactive effects, potentially altering the pathology of anemia.[51,60–63] For instance, coinfection between *Plasmodium* and *Schistosoma* was reported to have a multiplicative protective effects on anemia (in 5–18 years participants), with coinfected children having higher hemoglobin levels than children only infected with *P falciparum*.[62,64] Several other infections were also associated with anemia, namely viral and bacterial infections.[65]

It is also important to keep in mind that, while some parasites are the cause of micronutrient deficiencies, the nutritional status of the host can also influence the establishment of the infection itself and influence the pathway by which they will lead to anemia (dependent on the micronutrient that is lacking).[58,59,66] For instance, when facing a malaria infection, pre-established vitamin A deficiency may contribute to anemia by increasing the susceptibility to parasitemia, modulating iron metabolism, and impairing immune function, whereas vitamin E deficiency may lead to oxidant damage and consequent hemolysis. Moreover, folate and iron deficiency may impair erythrocyte synthesis, whereas riboflavin deficiency may decrease iron absorption and increase erythrocyte fragility, and zinc deficiency may impair immune function, increasing parasitemia, and leading to oxidative damage.[58] Interestingly micronutrient deficiencies can also protect against malarial anemia, specifically, vitamin E deficiency may protect from anemia by preventing antioxidant activity which increases the susceptibility of malaria parasites to oxygen radicals, whereas riboflavin deficiency may reduce parasite multiplication and growth, folate deficiency may impair the parasite metabolism, and iron deficiency may impair parasite multiplication.[58]

Nutrition-sensitive approach address mainly underlying and basic causes of anemia from a wide range of sectors, including disease control, water, sanitation, and hygiene and intersectoral strategies that address root causes (eg, poverty, lack of education, and gender norms). The control of parasitic infections aim both at stopping transmission (by treating infection) and also at reducing the morbidity that they cause (such as anemia). Anemia evaluation is suggested to be used as additional indicators to monitor population response to interventions in settings with high infection transmission due to relevant infection-anemia relationship.[53] In order to achieve the nutrition-sensitive approach aims, generally 3 main strategies are adopted: (1) pathogen-targeted chemotherapy that can bring direct relief from disease, promote child development, and help slow transmission; (2) long-term programs such as vector control; Water, Sanitation, and Hygiene (WASH) measures; or health education to change behavior that will help to prevent transmission; (3) a combination of both strategies.[7,53,67] The plausibility of those interventions is based on the assumption that in poor settings, where high prevalence, intensity, reinfection, incidence, and co-endemicity of anemia-related infections occur, there is an important contribution of micronutrient deficiency anemia related to infections, potentially refractory to supplementation and requiring multicausal resolution approaches.[7,23,68–71] In addition, in setting with a highly infectious profile, the screening and treatment of infections must be provided (especially in the case of malaria), to further prevent supplementation to adversely affect the infection-associated morbidity and mortality in children.[27,72–76]

One of the first infection to be considered is malaria, responsible for approximately 35% of anemia in African children, and whose control in endemic areas was reported to greatly reduce anemia and severe anemia (by >25% and by 60%, respectively).[16,23,25,77–81] When prevention is to be considered, chemoprevention and vector control are frequently planned. Intermittent preventive treatment comprehends the administration of sulfoxide-pyrimethamine, designed to protect children against clinical malaria and anemia in the first year of life, whereas vector control is the key intervention for global malaria control, aiming mainly at increasing the use of long-lasting insecticidal nets and indoor residual spraying to decrease transmission (among other less frequently used strategies).[7,16,77] The increased use of insecticide-treated nets has been reported to be successfully followed by significant decrease in the moderate-to-severe anemia among children aging between 6 and 23 months of age.[77]

Soil-transmitted helminths, schistosomiasis, and environmental/tropical enteropathy (caused by the ingestion of high amount of fecal bacteria), should also be considered, as they are associated to anemia (mainly IDA) as previously reported.[16,82–85] Therapeutic approaches, aiming at reducing the burden of soil-transmitted helminths infections and the intensity of infections, include single-dose, large-scale mass deworming, periodic preventive chemotherapy, or repeated large-scale administration of anthelminthic drugs to at-risk populations (preschool, school-aged children, and women of childbearing age).[86,87] For schistosomiasis, control includes large-scale drug treatment of at-risk populations (with praziquantel), snail control, improved sanitation, and health and hygiene education. Until recently, target populations included school-aged children and adults (with occupations involving contact with infested water) considered to be at risk or entire communities in endemic areas. New evidences of high prevalence among preschool children has, however, highlighted the need to include them in control strategies.[88–92] Furthermore, multisectoral strategies, targeting adequate water, sanitation, and hygiene practices may maximize and sustain the benefits of a decreased burden of those infections. For instance, promoting improvements in key hygiene behaviors (such as hand washing), treatment, and use of safe water, and using hygienic methods of disposal of feces and urine can be incorporated into health-sector activities, such as nutrition counseling, promotion, and assessments, as well as health visits.

Combining strategies to reduce anemia. Integrating therapeutic (deworming) and preventive (either food-based or WASH/malaria education) strategies can simultaneously treat infections and increase hematopoietic micronutrient intake or reduce disease transmission, which could result in the reduction of malnutrition and anemia.[7,31,33–40,69,86,87,93–97] For instance, deworming plus nutrition education has been reported to reduce anemia from 82.0% to 55.4%, while increasing green leafy vegetables consumption from 44.7% to 60.6%.[40] Furthermore, exclusive educational interventions targeting nutrition improvement; increased ability of mothers to identify malnutrition (from 15% to 99%); exclusive breastfeeding (79% vs 48% in control); weight gain (0.86 vs 0.77 kg in control); vegetables feeding; nutrient-dense foods at lunch (11% of difference between intervention and control groups); dietary requirements for energy, iron, and zinc; complementary feeding; and significantly reduced rates of stunting.[33–37,41,98] On the contrary, exclusive WASH educational interventions, health education on soil-transmitted helminths and schistosomiasis, significantly increased knowledge, reduced the prevalence of *A lumbricoides*

and *S mansoni*, and reduced the incidence of hookworms and the intensity of trichuriasis, ascaridiasis, and hookworms.[87,93] When deworming is added to WASH education, prevalence and intensity of soil transmitted helminths decrease and school children scored higher than control on their knowledge.[86,93] Studies evaluating educational approaches to malaria prevention showed improved knowledge regarding breeding sites, bed-net use and indoor spraying, increased windows and door net use and practice of maintaining clean environment and, a reduced number of reported malaria episodes and fever incidence.[94,95,99,100]

As mentioned, several comprehensive intervention strategies have correspondingly been tested. They, however, frequently evaluate the impact of education on nutrition or on infectious etiologies (whether or not combined with drug therapy) separately.[33–40,86,87,93–96] Thus, differences in their designs and methodologies hinder the face to face comparison and evaluation of results, rendering it difficult to define the best approach to reduce anemia. For instance, differences on the target population (adult or children), dimensions of the learning package (eg, for sanitation: stool disposal, water quality, or supply), the deliverers (community promoters/volunteers, teachers, local health workers, community leaders), the place where education occurs (at health center or at the communities), the number of intervention contacts, type of contacts (group meetings and/or individual contacts), and duration of the intervention/observation and mainly the combination with other strategies (micronutrient supplementation, construction of latrines, and hand-washing mechanisms, etc) can be observed.[33–40,86,87,93–96] Furthermore, one of the most inclusive studies, published recently by Humphrey et al[101] 2019, investigated the effect of 2 WASH interventions, either alone or combined with improved Infant and Young Child Feeding, concluding that education in nutrition reduce anemia and stunting, but adding WASH to the intervention had no major improvements on those effects. Similar results were reported by Null et al[102] 2018 within a cluster-randomized trial using interventive groups with several WASH and nutrition combinations. Neither study investigated the effect of combining deworming, nor was the impact of malaria preventive actions included.

Other aspects that need to be considered when designing interventions

Genetic causes of anemia. The normal adult hemoglobin molecule is composed of 4 globin subunits (2 α and 2 β), 1 heme pigment (bound to each globin chain) including 1 iron (II) atom in each heme pigment. Furthermore, >1000 human hemoglobin variants have been described in the literature, a number of which are abnormal types of hemoglobin.[27,103,104] These abnormalities may result from mutation in the globin structural genes (with consequent qualitatively or quantitatively abnormal hemoglobin), or from mutations in globin gene regulatory genes (with consequent production of lower quantity of hemoglobin). One of the most frequent variants is a single nucleotide A-to-T mutation in the structural β-globin gene resulting in sickle cell hemoglobin, named hemoglobin S, which arises from the glutamic acid-to-valine substitution at position 6 in the β subunit of hemoglobin.[26] Generally, individuals with 1 hemoglobin S gene are phenotypically normal (sickle cell trait) and people who inherit 2 hemoglobin S genes have sickle cell (or Hb SS) disease.[26] Sickle cell disease is associated with low hemoglobin values (7 or 8 g/dL), vaso-occlusive crises (which cause impaired blood flow and consequent local tissue hypoxia and even tissue/organ ischemia), long-term organ damage, and decreased survival.[26] On the contrary, quantitative alterations in globin gene expression may result in thalassemia. Hereditary RBC-enzymopathies can also cause anemia.[105–107] One of the more prevalent worldwide is glucose-6-phosphate dehydrogenase (G6PD) deficiency.[108–110] More than 300 genetic variants of G6PD have been described. Most of the mutations are, however, rare.[111] Hemolysis due to G6PD deficiency leads to decreased hemoglobin levels, and it is dependent on the class of the enzyme deficiency state and the magnitude of the oxidant impact.[26] The G6PD deficiency presents a geographic pattern, with the Class 3 variants (which are associated with mild to moderate enzyme deficit) being prevalent in populations of African ancestry.[26]

Physiologic and biologic aspects associated with anemia. The proportion of anemia attributable to the nutritional, infectious, and genetic causes discussed above may vary according to several physiologic and biologic aspects. In groups at high risk, multiple factors are frequently acting simultaneously to affect the risk of anemia. For instance, an imbalance between the nutrients needed for erythropoiesis and the higher nutrient demands for rapid growth, place children younger than 5 years at high risk for developing IDA.[16,112,113] Healthy-term infants are reported to be generally born with adequate iron stores which can last approximately 6 months, depending on maternal iron status during pregnancy.[8,26,114] It was reported that infants of anemic mothers were almost 6 times more likely to become anemic by the first year of life, even after adjusting for several confounding factors such as socioeconomic status, feeding practices, and morbidity.[8] However, while generally considered adequate for the first 4 to 6 months of life, it should be considered that the amount of iron in breast milk may decrease through the course of breastfeeding period and may not be absorbed as efficiently as predicted.[8,115–117] Sequentially, the introduction of complementary foods should have appropriate quantity and bioavailability of iron and low levels of inhibitors of iron absorption, which could reduce the absorption.[42,115,118,119] On the contrary, male infants may be at greater risk of lower hemoglobin concentrations and/or poor iron status as compared to female infants.[16,120,121] An assessment of iron stores in infants during the first year of life found that male infants had consistently lower iron stores and estimated body iron, and higher rates of iron deficiency than female infants.[120,122,123] It was suggested that in utero factors (potentially hormonal), could be influencing the status of iron at birth and also the erythropoietic activity.[16] Furthermore, there are reports of high heterogeneity regarding the hemoglobin levels that should define anemia among different countries, particularly in tropical regions.[124–126]

Health determinants of anemia. The prevalence and distribution of anemia (particularly IDA) is considered to have (1) fundamental, (2) underlying, (3) intermediate, and (4) immediate determinants, involving a complex interplay of factors. (1) Fundamental determinants involve socioeconomic, political, climatic, and environmental conditions; (2) underlying determinants relate to agricultural factors (food/cash flow, crop yields, livestock), economic circumstances (regional/local wealth, equity/equality, literacy/education), and health policies (health coverage/insurance, control programs, fortification policies); (3) intermediate determinants relate to food availability (food security, household income/allocation/wealth, access to cereals/vegetables/meat, food fortification, access to fortified complementary foods, meal/dietary patterns) and health care (access to health care, access to supplementations, health worker

knowledge, sanitation and hygiene); (4) immediate determinants relates to nutritional micronutrient intake (iron content of food, iron availability, heme/nonheme content, consumption of inhibitors and enhancers), blood loss due to infections, and genetics.[27,52]

From a general perspective, socioeconomic, behavioral, and environmental determinants can put some individuals and population groups at a higher risk of anemia.[7,27,52] Socioeconomic status affects the prevalence of anemia through several pathways. For instance, poverty is associated with poor housing, water, sanitation and hygiene, and inadequate infrastructure that may lead to increased disease risk, adverse nutrition behaviors (such as poor dietary practices and poor dietary quality), and inadequate access to anemia prevention and treatment services (such as iron supplements, deworming, and insecticide treated bed nets). Women and children in the lowest wealth quintiles were found to have 25% and 21% higher risk of anemia, than those in the highest wealth quintiles.[16] On the contrary, the low maternal education level may affect mother's ability to access and understand health and nutrition information, negatively affecting their children's quality of diet, or influence decision-making and compliance with recommended health and caretaking practices, being frequently associated with anemia.[5,83,127] It has been also reported that some populations belonging to specific settings or ethnic, cultural, or religious groups may be at a greater risk of anemia due to having less opportunities for generating income, accessing education, health care, social services, water, sanitation, and hygiene, but also to being differentially exposed to infectious, having different dietary patterns, or a different genetic background.[8,16] Gender inequality and cultural practices may also play a role in the development of anemia in children, occurring either when mothers face increased physiological risk (in turn associated with the factors mentioned above) or when the child feeding practices lead to differential risk of anemia between boys and girls.[16]

Conclusion

In Angola, the latest data on the prevalence of anemia in children younger than 5 years is worrisome. In addition, there is lack of reports regarding the main associated factors and there are no specifically designed interventions to address it. It should be considered that the internal results from government or other institution's studies may not be available and therefore are inaccessible. Nevertheless, the prevalence reported is indicative of a severe public health problem, but also have geographic heterogeneity and was frequently associated with infections. We did not found reports on the role of child feeding practices on the occurrence of anemia. To design an intervention in this misinformed scenario would result in unsustain spenditure of resources. Nevertheless, several interventions targeting nutritional anemias have already been tested in Africa and could be used as template. In this study, the rational and results of some important interventional strategies conducted in Africa have been resumed. We strongly recommend that educational plus therapeutic strategies, combining nutrition-specific and nutrition-sensitive approaches are considered.

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Conflicts of interest

The authors declare no competing interests.

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Paper II: **Iron Deficiency Anaemia among 6-to-36-month children from northern Angola**

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Iron deficiency anaemia among 6-to-36-month children from northern Angola

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Abstract

Background: Angola is one of the southern African countries with the highest prevalence of anaemia. Identifying anaemia determinants is an important step for the design of evidence-based control strategies. In this study, we aim at documenting the factors associated with Iron Deficiency Anaemia (IDA) in 948 children recruited at the Health Research Center of Angola study area during 2015.

Methods: Data on demographic, socio-economic and parental practices regarding water, sanitation, hygiene, malaria infection and infant and young child feeding were collected, as well as parasitological, biochemical and molecular data. Total and age-stratified multivariate multinomial regression models were fitted to estimate the magnitude of associations between anaemia and its determinants.

Results: Anaemia was found in 44.4% of children, of which 46.0% had IDA. Overall, regression models associated IDA with age, gender and inflammation and non-IDA with age, zinc deficiency and overload, *P. falciparum* infection, sickle cell trait/anaemia. Among 6-to-23-month-old children IDA was associated with continued breastfeeding and among 24-to-36-month-old children IDA was associated with stunting. Furthermore, zinc deficiency was associated with non-IDA among both age groups children. Inflammation was associated with IDA and non-IDA in either 6-to-23 and 24-to-36 months old children.

Conclusion: The main variables associated with IDA and non-IDA within this geographic setting were commonly reported in Africa, but not specifically associated with anaemia. Additionally, the associations of anaemia with inflammation, zinc deficiency and infections could be suggesting the occurrence of nutritional immunity and should be further investigated. In age groups, zinc overload was observed to protect under 6 months children from Non-IDA, while continued breastfeeding was associated with increased IDA prevalence in 6-to-23 months children, and stunting was suggested to increase the odds of IDA in 24-to-36 month children. This site-specific aetiology profile provides an essential first set of evidences able to inform the planification of preventive and corrective actions/programs. Nevertheless, regional and country representative data is needed.

Keywords: Iron deficiency anaemia, Aetiologies, Preschool children, Northern Angola

Background

Several studies have summarized the worldwide prevalence of anaemia, reported to be 30% in 1985, 33.3% in 1990, 32.9% in 2010 and 27.0% in 2013 [1–8]. Kassebaum et al reported that globally the prevalence dropped between 1990 and 2010/2013, as well as the number of countries with prevalence higher than 50% (from 20 to 4 countries) [1, 4, 9, 10]. In Angola, the southern African country with the highest prevalence in 1990, a similar tendency of decreasing prevalence was reported (from 50 to 60% in 1990 to 40–50% in 2013 for all ages) [4]. In 2010, the regional prevalence of anaemia in children was reported to be 21.6% in the south and 57% in the north of the country. A national multiple indicators survey,

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conducted between 2015 and 2016, reported that 65% of 6 to 59 months were anaemic, and the prevalence was higher in 6-to-11 months children (83% in 6–8 months and 82% in 9–11 months children) and that higher severity occurred in 12–17 months children [11–13].

Despite being the single most important cause of anaemia and anaemia-related disability, the contribution of iron deficiency showed a modest decrease (from 66.2 to 62.6% between 1990 and 2013) [4]. Besides iron deficiency, hookworm, sickle cell disorders, thalassaemias, schistosomiasis, and malaria were also important causes, although showing substantial variability with age, gender, and geography [1, 4]. For instance, the most relevant cause-specific prevalence of anaemia in Western and Central sub-Saharan Africa were reported to be malaria and haemoglobinopathies, which collectively explained 80% of anaemia cases [4]. In Angola, anaemia has been associated with undernutrition (responsible for 13% of the anaemia cases), but also with infections, namely by *Hymenolepis nana*, *Plasmodium falciparum* and *Schistosoma haematobium* [13, 14]. The last 2 parasites were reported to be responsible for 16 and 10% of the anaemia cases in children living in the Dande municipality, respectively [13, 14]. According to the few existing studies regarding the aetiologic profile of anaemia in Angola, undernutrition and infections are important contributors to the total burden in the country, although micronutrient deficiencies have not been fully explored [13, 14]. Associations between nutritional and infectious aetiologies should be further investigated considering their relevance and that no published data is currently available for this setting. For instance, nutritional anaemias are reported to be directly linked to micronutrient deficiencies, which in turn can be associated with underlying, intermediate and/or immediate causes of malnutrition [9, 15]. However, infections can also cause anaemia indirectly through micronutrient deficiencies, despite that other mechanisms may cause non-nutritionally related anaemias (such as malabsorption, chronic blood loss, anorexia, inflammation or haemolysis) [15, 16].

From a public health point of view, a context-specific aetiologic profile should be determined in order to design the appropriate preventive, control or treatment strategies [17]. For instance, the coexistence of iron deficiency and malaria may highlight the paradox for anaemia control, as iron supplementation was suggested to increase malaria risk, and the infection was recommended to be screened and treated before supplementation [18, 19]. Additionally, the attributable weight of hereditary causes, such as sickle cell anaemia and Glucose-6-phosphate dehydrogenase deficiency, should also be investigated, as they may in turn be directly associated with the occurrence of total anaemia, or influence the occurrence of other causes mentioned above [15, 20, 21].

In the present study, considering that iron deficiency anaemia (IDA) is the endpoint for several direct and indirect causal pathways, and that it is reported to play a major role on the total burden of anaemia, we aim at documenting key basic, intermediate, and immediate nutritional determinants of IDA, accounting also for the contribution of hereditary haemolytic factors.

Methods

Study design and sampling

The sampling strategy, chosen for this observational cross-sectional study, was a non-probabilistic (convenient) sampling. First, we identified administratively and geographically isolated hamlets with functional health posts (i.e., providing daily primary care), in turn located within the CISA's HDSS study area. Then, all under 3 years old children resident in those hamlets were listed and invited to participate, using a census approach. The criterion to define eligible hamlets was based in the higher facilities in mobilizing the population and logistical advantages associated with health posts, while the census approach was adopted because variations in the density of eligible children estimated by CISA's HDSS database, were expected and the real density in each cluster was needed.

Study site and population

Resulting from this sampling strategy, seven hamlets with functional health posts were selected from the CISA's Health and Demographic Surveillance System (HDSS) study area [22]. CISA's 4700 km2 study area, comprehend mostly 3 communes from the Dande municipality in the Bengo Province, where the demographic and economic aspects of their 15,579 households and 59,635 residents (registered initially) are being followed since 2009 and where several studies have been conducted [13, 14, 22–28]. In average, each household of that area have 3.8 inhabitants (4.2 in urban and 3 in rural areas), that live frequently in houses made mainly by adobe walls, iron sheets roofs, without kitchen (near 70% of the houses) and without latrines (or having to chare them) [23]. Drinking water was reported to be obtained mainly from an unimproved source, namely from rivers (48%), unprotected dug well (10%) and/or lakes and irrigation channels (3%) [23]. Additionally, bed-net coverage (25.1%) and history of previous treatment for *S. haematobium* and Geohelminth infections in preschool children were reported to be low in 2010 (3.5 and 15.9%, respectively). Contrasting with the prevalence of being infected with at least one or 2 geohelminth infection was 22.6 and 3.8%, respectively, at the same period [13, 14].

For this study, all under-3-year-old children and their mothers/caregivers, resident in the selected hamlets were considered eligible, being listed and invited to participate (using a census approach).

After explaining the study's objectives, and obtaining verbal acceptance to participate, the field technician delivered a "participant information form" and a stool container to eligible families and instructed them to be present at the health center for evaluation the following day. At the end of the census approach, 1106 households were considered eligible and were invited to participate. Of those, 830 primary caregivers (mainly the children's mothers) attended to the evaluation day at the health centers and signed an informed consent. In total, 948 children were evaluated. Approximately half of the children with evaluable data were aged between 6 and 23 months: 517/943 (54.9%) with a similar proportion of boys (50.6%, 479/946) and girls (49.4%, 467/946). Additionally, one third of the children lived in a household with 4 or 5 more residents (35.3%, 335/948), and close to half lived with another under 5-years old child (48.5%, 330/680).

Training

For this study, 6 field workers and 2 nurse technicians (nurse's aide or assistant) were selected and trained. The training course comprehended theoretical lessons on: 1) introduction to research questions, 2) study goals and design, 3) basic concepts regarding the diseases studied, 4) mobilization techniques, 5) methodologies for data collection (specific structured questionnaire interviews, anthropometric evaluations, recognition of signs and symptoms of micronutrient deficiency and temperature measurement). Nurse technicians undergone an additional 3-day training on: 1) best practices for drug administration to young children and 2) domiciliary treatment and 3) support to physician in hospital-based consultations.

Sample and data collection

A standardized questionnaire was administered to caregivers. Data was collected regarding demographics (age, gender, household size and number of under 5 children household residents) socio-economic (monthly income, daily expenditure with food and water, ownership of latrine, crop field and bednet and activities of hunting or breeding animals) and parental practices (water sanitation and hygiene (WASH), malaria and Infant and Young Child Feeding (IYCF)) [29, 30]. The monthly income, daily expenditure with food and water were analysed based on the cut-offs of 15,000 AKZ (approx. 40 EUR), 1000 AKZ (approx. 3 EUR) and 200 AKZ (approx. 0.6 EUR), respectively. Furthermore, the proportion of children in exclusive breastfeeding (regarding children under 6 months who were reported to have received only breast milk), in continued breastfeeding (children over 5 months who were both breastfed and complementary fed), that have achieved the individual Minimum Dietary Diversity (MDD, to those older than 5 months who consumed 4 or more foods from the groups: 1) grains, roots and tubers, 2) legumes and nuts, 3) dairy products, 4) flesh foods, 5) eggs, 6) vitamin A-rich fruits and vegetables, and 7) other fruits and vegetables), who consumed haeme-iron (animal based foods, mainly organs and meat, poultry, eggs and fish) and non-haeme iron rich foods (plant-based foods, mainly legumes and dark green leafy vegetables), were classified as previously described, using 24 h recall data [30, 31].

Weight, measured in electronic or platform scales, height (measured in standardized infantometer or stadiometers) and oedema, were collected and used to calculate the anthropometric indices to classify malnutrition (either in children's and their caregivers), following WHO guidelines [32]. Mid-Upper Arm Circumference (MUAC) was used to classify acute malnutrition and to refer children to the emergency unit of Bengo's General Hospital. Peripheral blood was collected on site according to WHO guidelines to good phlebotomy practice [33]. The blood samples for iron, zinc and C-reactive protein (CRP) determination were collected into Micro tubes 1.1 ml Z-Gel® (Sarstedt, Nümbrecht, Germain), then centrifuged to separate serum, which in turn was stored at − 20 °C until processing. Blood samples for molecular analysis were collected on filter paper, air dried and stored until processing. Stool and urine samples were obtained on or around the evaluation day, with exception of some younger children incapable to verbalize urge to urinate, in which a paediatric urine collection bag was applied. Formalin (10%) was added to stool samples, and along with urine samples, were stored in a thermal box with coolers for transportation to the lab (no more than 4 h).

Laboratorial analyses

Parasitological analysis comprised the diagnosis of *P. falciparum* and *P. vivax* malaria, performed using a rapid diagnostic test (SD BIOLINE Malaria Ag P.f/P.v®, Standard Diagnostics, Inc., Republic of Korea) according to the manufacturer guidelines. Diagnosis of intestinal parasites were performed using Kato-Katz technique and Parasitrap® kits (Biosepar, Germany) and urogenital schistosomiasis was diagnosed by urine filtration, using Whatman® Nuclepore™ membranes (diam. 25 mm, pore size 12 μm, polycarbonate, Merck, Germany) [34–36]. Biochemical analysis included determining blood levels of haemoglobin using an Hemocue® Hb 301 System (Angelholm, Sweden), CRP serum levels, ferritin and zinc, using an automated autoanalizer (BT1500, Biotecnica Instruments S.p.A, Rome, Italy) and CRP turbidimetric latex®, Ferritin® and Zinc® kits (Quimica Clínica Aplicada S.A., Tarragona, Spain). Molecular analyses comprehended DNA extraction using InstaGene™ Matrix (Bio-Rad laboratories, Inc. United States of América),

screening for sickle cell anaemia and sickle cell trait (by PCR-RFLP), and G6PD deficiency (by rtPCR) [37, 38].

Children were considered anaemic if haemoglobin (Hb) levels were below 11.0 g/dL with the following stratification: mild anaemia if Hb was between 10.0 and 10.9 g/dL, moderate anaemia if Hb was between 7.0 and 9.9 g/dL and severe anaemia if Hb was lower than 7.0 g/dL [5, 39, 40]. Iron-deficiency was considered to be present if serum levels of ferritin were below 12 μg/L in the absence of inflammation or below 30 μg/L if inflammation (serum CRP levels higher than 5 mg/L) was present [41]. IDA was considered when Hb level was below 11.0 g/dL and ferritin deficiency was also observed. Pathological zinc levels were considered whenever, serum levels were bellow 70.0 μg/dL (Zinc deficiency) or above 150.0 μg/dL (Zinc overload) [42].

Prevalence of the studied parasites was determined as the proportion between all infected children and all children delivering the correspondent sample. Children were considered to have diarrhoea if caregivers reported that the children had at least one episode of 3 or more aqueous dejections per day in the last 2 weeks. Z- scores of weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ) were determined using WHO Anthro software (version 3.2.2) for children and body mass index (BMI) was calculated and used to classify undernutrition in their mothers (considered to be eutrophic if BMI 18.50–24.99 kg/m^2, undernourished if BMI < 18.50 kg/m^2 and overnourished if BMI > 25 kg/m^2 [43].

Statistics

In this study, 95% confidence intervals (CI95) were estimated for the prevalence's. Crude multinomial models were fitted, each with a single independent variable and taking children without anaemia as the reference category of the dependent variable (vs. IDA and non-IDA anaemia). Variables that in those models were significantly associated with any type of anaemia, considering a significance level of 10% ($p < 0.10$), were then included as independent variables in a multivariate multinomial model. For those models, the manual stepwise method was used to retain only the variables with an association with anaemia, at a significance level of 5% ($p < 0.05$) in the final model. Models considering all children and stratified by age groups (children under 6 months, between 6 and 23 months and between 24 and 36 months) were fitted. Nagelkerke R square was used to evaluate the goodness of fit of the models.

Results

Nutritional status of children and their feeding practices

The prevalence of moderate to severe undernutrition was as follows: 9.9% wasting, 26.7% stunting and 20.3% underweight. Anaemia was present in 44.4% of children, 46.0% of which were diagnosed with IDA. Serum levels of ferritin, corrected for inflammation, showed 38.1% of additional iron deficient children.

Regarding the feeding practices, we found that 49.3% of the under 6 months children reported to be exclusively breastfeed in the previous 24 h. Also, 52.5% of children with 6 or more months were breastfed and complementary fed. The Minimum Dietary Diversity (MDD) for continued breastfed children was lower (11.4%, 72/633) than children being only complementary fed (14.2%, 93/633). Many of the children that did not meet the MDD were found to consume mainly foods from 2 or 3 food groups (36.2% (203/561) and 56.7% (318/561), respectively). Haeme-iron and non-haeme iron rich foods were reported to have been consumed by 75.8 and 35.3% of the children aging 6 or more months of age (Table 1).

We observed that 39.4% (186/472) of the caregivers reported to spend more than 200 AKZ per day in water, while 33.5% (292/871) reported to spend more than 1000 AKZ per day in food. Also, 40.1% (371/927) reported to being subsistence farmers and 26.6% (246/924) reported breeding or hunting animals (Table 1).

Infectious state of children and mother-to-children infection preventive practices

Within the children with evaluable data, 45.3% had CRP levels consistent the occurrence of inflammatory processes. Of those, 3.3% had malaria (considered here as malarial inflammation) and 42.0% were considered non-malarial inflammation. Furthermore, the prevalence of *P. falciparum*, *A. lumbricoides*, *G. lamblia* and *S. haematobium* was 5.2, 3.8, 7.5, 6.3 and 15.2%, respectively. Despite being less prevalent, *T. trichiura* (0.5%, 4/787), *E. histolytica* (0.3%, 2/787), *S. mansoni* (0.1%, 1/787), *H. nana* (0.8%, 6/787) and *S. stercoralis* (0.5%, 4/787) were also observed. Diarrhoea in the 2 weeks prior to evaluation was reported in 41.2% of the children. Eggs from hookworms were not observed, either by Kato-Katz or Parasitrap.

There were bednets in 50.6% (470/929) of the households and 42.8% (391/913) of the children had slept under the bednet in the previous night. Furthermore, 73.5% (685/932) of the caregivers reported to treat the drinking water, which 50.4% (467/927) was obtained from natural sources. The water used for bathing was also reported to be mainly obtained from unimproved sources (63.1%, 502/796). Despite that, 74.4% (694/933) of the caregivers reported to have latrine, 35.0% (327/935) reported to deposit the stool in open sky when outside, while the majority reported to deposit in latrines or bury the stool. We also observed that 70.5% (589/835) of children were wearing shoes at the evaluation moment.

Genetic features of children

Regarding genotyping, we observed that 42.5% (195/459) of the females had at least one G6PD polymorphism (B/

Table 1 Characterisation of study children: demographics, nutritional status, Infant and Young Child Feeding practices, infections and infection preventive practices and genetic features

Variables	Categories	n	N	Estimated proportion (95% CI)
Demographic characteristic of children				
Age (in months)	< 6 months	155	948	16.4 (14.1–18.8)
	6–23 months	520	948	54.9 (51.7–58)
	24–36 months	273	948	28.8 (26–31.8)
Gender	Female	459	948	48.4 (45.2–51.6)
	Male	489	948	51.6 (48.4–54.8)
Nutritional status and feeding practices				
Anaemia	No	527	912	57.8 (54.6–61)
	IDA	177	912	19.4 (17–22.1)
	Non-IDA	208	912	22.8 (20.2–25.6)
Zinc deficiency	Yes	58	687	8.4 (6.6–10.8)
Zinc overload	Yes	165	794	20.8 (18.1–23.7)
Wasting	Moderate to severe	93	942	9.9 (8.1–11.9)
Stunting	Moderate to severe	252	943	26.7 (24–29.6)
Underweight	Moderate to severe	191	943	20.3 (17.8–22.9)
Exclusive breastfeeding (< 6 months)	Yes[a]	74	150	49.3 (41.4–57.3)
Continued breastfeeding (6 to 36 months)	Yes[a]	413	786	52.5 (49–56)
Minimum Dietary Diversity (6 to 36 months)	Yes[a]	165	726	22.7 (19.8–25.9)
Non-haeme Iron rich foods (6 to 36 months)	Yes[a]	256	726	35.3 (31.9–38.8)
Haeme Iron rich foods (6 to 36 months)	Yes[a]	550	726	75.8 (72.5–78.7)
Feeding frequency (6 to 36 months)	0–1 times	73	703	10.4 (8.3–12.9)
	2–3 times	389	703	55.3 (51.6–59)
	> = 4 times	241	703	34.3 (30.9–37.9)
Infections and infection preventive practices				
P. falciparum	Yes	49	946	5.2 (3.9–6.8)
At least one intestinal/urogenital parasite	Yes	127	833	15.2 (13–17.8)
A. Lumbricoides	Yes	30	787	3.8 (2.7–5.4)
G. lamblia	Yes	59	783	7.5 (5.9–9.6)
S. haematobium	Yes	36	570	6.3 (4.6–8.6)
Diarrhoea in the last 2 weeks	Yes[a]	386	938	41.2 (38–44.3)
Inflammation (CRP)	No	465	850	54.7 (51.3–58)
	Malarial inflammation	28	850	3.3 (2.3–4.7)
	Non-malarial inflammation	357	850	42 (38.7–45.3)
Sleeping under the bednet in the previous night	Yes	391	913	42.8 (39.7–46.1)
Treated drinking water	Yes	685	932	73.5 (70.6–76.2)
Main source of drinking water	Unsafe (river, lagoon)	467	927	50.4 (47.2–53.6)
	Safe (piped, fountain)	460	927	49.6 (46.4–52.8)
Main source of water for bath	Unsafe (river, lagoon)	502	796	63.1 (59.7–66.3)
	Safe (piped, fountain)	294	796	36.9 (33.7–40.3)
Place for faecal disposal	Unsafe (open sky)	327	935	35 (32–38.1)
	Safe (latrine or buried)	608	935	65 (61.9–68)
Wearing shoes at evaluation	Yes	589	835	70.5 (67.4–73.5)

Table 1 Characterisation of study children: demographics, nutritional status, Infant and Young Child Feeding practices, infections and infection preventive practices and genetic features *(Continued)*

Variables	Categories	n	N	Estimated proportion (95% CI)
Genetic features				
G6PD genotype of females	B/B, A/A, B/A	264	459	57.5 (53–62)
	B/A-, A/A-, A−/A-	195	459	42.5 (38–47)
G6PD genotype of males	B, A	271	479	56.6 (52.1–60.9)
	A-	208	479	43.4 (39.1–47.9)
Sickle cell (HBB genotype)	AA	629	848	74.2 (71.1–77)
	AS	203	848	23.9 (21.2–26.9)
	SS	16	848	1.9 (1.2–3)

[a] Definition is described in Methods

A- (29.6%), A/A- (8.3%) and A−/A- (4.6%)), while a similar prevalence occurred in males,43.4% (208/479). In addition, 23.9% (203/848) of the children were found to have the sickle cell trait and 1.9% (16/848) were homozygous for sick cell anaemia (Table 1).

Characteristics of caregivers

Caregivers were mainly young adults with ages between 20 and 39 years, followed by adolescents (under 20 years old) and older adults (above 40 years old). The majority were the children's mothers, married or living with their partner and reporting to have attended school. Additionally, the mothers' anthropometric measures according to their Body Mass Index (BMI) revealed that 59.7% had an adequate nutritional status, while 34.1% were overweight and 6.2% underweight (Table 2).

Factors associated with IDA and non-IDA

In crude multinomial models, and compared with children without anaemia, the occurrence of IDA was associated with age (OR:11.1, 95%CI: 4.42–27.96 for 6–23 months children and OR:3.5, CI: 1.31–9.20 for 24–36 months), gender (OR:1.9, CI: 1.33–2.69 for males), having intestinal/ urogenital parasite (where children with at least one studied parasite appearing to be less likely to have IDA than children without any parasite, OR:0.5, CI: 0.28–0.90), and having inflammation (OR:4.7, CI: 1.65–13.43 for inflammation plus malaria infection and OR: 2.4, CI: 1.67–3.44 for inflammation without malaria infection). The same models suggested that Non-IDA was associated with the school level of caretakers (OR:1.8, CI: 1.02–3.21 for those achieving the primary level, when compared to those without school frequency), source of drinking and bath water (OR:0.7, CI: 0.48–0.91 and OR: 0.6, CI: 0.44–0.93, respectively, for artificial/improved sources), zinc levels with children with zinc deficiency having higher odds of having Non-IDA than children with normal values (OR:2.8, CI: 1.56–5.19), and children with zinc overload being less likely to have Non-IDA than children with normal levels (OR:0.6, CI: 0.38–0.95)), malarial inflammation (OR:4.6, CI: 1.79–11.83), *P. falciparum* infection (OR: 3.2, CI: 1.63–6.21), and both sickle cell trait and sickle cell anaemia (OR:1.6, CI: 1.05–2.27 and OR:17.7, CI: 3.91–80.22, respectively).

Furthermore, crude multinomial age-stratified analysis showed that among children under 6 months,

non-IDA was associated with age (OR:1.3, CI:1.067–1.591) and with having zinc overload (where children with zinc overload had significantly less Non-IDA than under 6 months children with normal zinc levels (OR:0.3, CI:0.13–0.79)). Unfortunately, numeric problems didn't allow to investigate associations with IDA. Furthermore, children aged between 6-to-23 months were more likely to be diagnosed with IDA if they were males (OR:2.3, CI:1.48–3.46), being continued breastfeeding (OR:1.7, CI:1.05–2.82) and if they had inflammation without malaria (OR:2.3, CI:1.46–3.50). These associations weren't observed to occur regarding the diagnosis of Non-IDA. Nevertheless, in this age group, the diagnosis of Non-IDA was more likely to occur among children living in households with one additional children under 5 (OR:2.4, CI:1.15–4.82, comparatively to none), *P. falciparum* infection (OR:5.4, CI:1.98–14.94), inflammation with malaria (OR:8.3, CI:2.16–31.99) and/or having sickle cell anaemia (OR:20.2, CI:2.44–167.49, comparatively to having a normal genotype or having the sickle cell trait), associations that weren't observed for children in the same age group with IDA. In older children (aging between 24 and 36 months) the occurrence of IDA appeared to be associated with the number of residents in the same household (OR: 0.4, CI:0.15–0.83, for living with more than 5 residents), children being moderate-to-severely stunted (OR: 2.5, CI:1.14–5.50) and having inflammation (OR:4.3, CI: 1.69–11.02). Similarly, to the previous age group, children aging between 24 and 36 months that had zinc deficiency were also more likely to have Non-IDA than children with normal zinc levels (OR:3.1, CI:1.31–7.52),

When all variables with significant associations with either IDA or Non-IDA were added to a multivariate multinomial regression model, only age, gender and

Table 2 Characterisation of households and caregivers of studied children

Variables	Categories	n	N	Estimated proportion (95% CI)
Household characteristics				
Estimated monthly income (AKZ)	< 15,000	356	602	59.1 (55.2–63)
	≥ 15,000	246	602	40.9 (37–44.8)
Daily food expenditure (AKZ)	< 1000	579	871	66.5 (63.3–69.5)
Median = 1000.0; Mean = 1361.2; SD = 2486.5	≥ 1000	292	871	33.5 (30.5–36.7)
Daily water expenditure (AKZ)	< 200	286	472	60.6 (56.1–64.9)
Median = 200.0; Mean = 368.7; SD = 1095.8	≥ 200	186	472	39.4 (35.1–43.9)
Latrine ownership	Yes	694	933	74.4 (71.5–77.1)
Bednet ownership	Yes	470	929	50.6 (47.4–53.8)
Ownership of land for agriculture	Yes	372	927	40.1 (37–43.3)
Breeding or hunting animals	Yes	246	924	26.6 (23.9–29.6)
Number of residents	<= 3	137	948	14.5 (12.4–16.8)
Median = 6.0; Mean = 5.9; SD = 2.5	4–5	335	948	35.3 (32.4–38.4)
	6–7	262	948	27.6 (24.9–30.6)
	> = 8	214	948	22.6 (20–25.3)
Number of children under 5 years old	None	170	680	25 (21.9–28.4)
Median = 1.0; Mean = 1.2; SD = 1.2	1	330	680	48.5 (44.8–52.3)
	> = 2	180	680	26.5 (23.3–29.9)
Characteristics of the caregivers				
Age	< 20 years	150	861	17.4 (15–20.1)
Median = 27.0; Mean = 27.6; SD = 8.5	20–39 years	643	861	74.7 (71.7–77.5)
	> 40 years	68	861	7.9 (6.3–9.9)
Gender	Male	35	828	4.2 (3.1–5.8)
	Female	793	828	95.8 (94.2–96.9)
Marital status	Married or living with partner	660	817	80.8 (77.9–83.3)
	Single, divorced or widow	157	817	19.2 (16.7–22.1)
School frequency	Yes	701	804	87.2 (84.7–89.3)
Education level achieved	Primary level	238	655	36.3 (32.7–40.1)
	Basic level	330	655	50.4 (46.6–54.2)
	High school to university	87	655	13.3 (10.9–16.1)
Number of children under 5 years old in the household	<=2	344	813	42.3 (39–45.7)
Median = 3.0; Mean = 3.3; SD = 2.0	3–4	265	813	32.6 (29.5–35.9)
	> = 5	204	813	25.1 (22.2–28.2)
Nutritional status of mothers[a]	Eutrophic (BMI 18.50–24.99 kg/m^2)	478	801	59.7 (56.2–63)
	Underweight (BMI < 18.50 kg/m^2)	50	801	6.2 (4.8–8.1)
	Overweight and obese (BMI > 25 kg/m^2)	273	801	34.1 (30.9–37.4)

[a]Only non-pregnant mothers were included

inflammation sustained the statistical significance association with IDA, suggesting that children 6-to-23 months had higher probability of having IDA than under 6 months children, similarly for males comparatively to females and non-malarial inflammation comparatively to children with no inflammation, while *P. falciparum*, sickle cell trait and sickle cell anaemia sustained their significantly association with Non-IDA, with age becoming also significantly associated (Table 3).

In the age-stratified adjusted models we found that Non-IDA in under 6-month children was associated with age and zinc overload. Furthermore, among 6-to-23 months children, the occurrence of IDA sustained its association with gender, being continued breastfeed and

Table 3 Multinomial multivariate regression models for IDA and non-IDA

Independent variables		Non anemic	IDA	*p*	Non-IDA	*p*
			OR (IC95%)		OR (IC95%)	
Total population (1)						
Age	< 6 months	1	Ref		Ref	
	6–23 months		7.4 (2.87, 19.11)	< 0.001	0.7 (0.43, 1.15)	0.166
	24–36 months		2.0 (0.73, 5.53)	0.180	0.5 (0.27, 0.80)	0.006
Children's gender	Female	1	Ref		Ref	
	Male		2.0 (1.32, 2.91)	0.001	1.3 (0.87, 1.81)	0.216
Zinc	Normal	1	Ref		Ref	
	Deficiency		1.6 (0.67, 3.61)	0.306	3.2 (1.64, 6.25)	0.001
	Overload		0.8 (0.47, 1.26)	0.300	0.6 (0.36, 0.96)	0.033
P. falciparum	No	1	Ref		Ref	
	Yes		1.3 (0.26, 6.81)	0.733	3.1 (1.05, 9.42)	0.041
Inflammation	No	1	Ref		Ref	
	Malarial Inflammation		3.8 (0.56, 25.70)	0.174	1.8 (0.44, 7.36)	0.415
	Non-malarial Inflammation		2.4 (1.62, 3.65)	< 0.001	1.3 (0.90, 1.94)	0.157
Sickle cell (HBB genotype)	AA	1	Ref		Ref	
	AS		1.00 (0.59, 1.55)	0.853	1.6 (1.03, 2.35)	0.035
	SS		1.2 (0.10, 13.54)	0.904	16.6 (3.56, 77.04)	< 0.001
Children under 6 month (2)						
Age	Continuous variable	1	–	–	1.3 (1.02, 1.57)	0.031
	Normal	1	–	–	Ref	
Zinc	Deficiency		–	–	1.1 (0.20, 5.85)	0.927
	Overload		–	–	0.3 (0.12, 0.73)	0.008
Children 6 to 23 months (3)						
Gender	Female	1				
	Male		2.1 (1.34, 3.27)	0.001	1.3 (0.78, 2.10)	0.321
Continued breastfeeding	No	1				
	Yes		1.9 (1.11, 3.13)	0.019	1.6 (0.92, 2.90)	0.095
Zinc	Normal	1				
	Deficiency		1.4 (0.42, 4.48)	0.604	4.4 (1.55, 12.28)	0.005
	Overload		0.8 (0.44, 1.30)	0.307	0.7 (0.35, 1.27)	0.221
Inflammation	No	1				
	Malarial Inflammation		2.3 (0.43, 11.95)	0.331	9.1 (2.34, 35.71)	0.001
	Non-malarial Inflammation		2.2 (1.42, 3.47)	< 0.001	1.5 (0.90, 2.48)	0.119
Children 24 to 36 months (4)						
Age	Continuous variable	1	0.9 (0.77, 0.98)	0.020	1.0 (0.955, 1.13)	0.408
Zinc	Normal	1				
	Deficiency		1.4 (0.38, 5.28)	0.609	3.6 (1.41, 9.09)	0.007
	Overload		1.0 (0.26, 4.14)	0.960	0.7 (0.23, 2.35)	0.605
Stunting	Normal	1				
	Moderate to severe		2.6 (1.09, 6.20)	0.031	1.2 (0.60, 2.23)	0.675
Inflammation	No					
	Malarial Inflammation	1	18.2 (3.55, 92.76)	< 0.001	2.3 (0.54, 9.94)	0.262
	Non-malarial Inflammation		4.0 (1.45, 11.01)	0.007	0.4 (0.21, 0.90)	0.024

Only variables with a significance level of 10% ($p < 0.10$) were included as independent variables in a multivariate multinomial model [1]. Variables excluded from the model ($p > 0.05$): educational level of the caregiver, breeding or hunting animals, main source of drinking and bath water and being infected with at least one intestinal or urogenital parasite. Model adjustment: Pearson: $\chi2(170) = 162.9$, $p = 0.638$; Deviance: $\chi2(170) = 169.9$, $p = 0.488$; R2 Nagelkerke = 0.208 [2]. Variables excluded from the model ($p > 0.05$): Inflammation (Non-malarial inflammation). Model adjustment: Pearson: $\chi2(22) = 43.1$, $p = 0.500$; Deviance: $\chi2(22) = 48.6$, $p = 0.100$; R2 Nagelkerke = 13.5% [3]. Variables excluded from the model ($p > 0.05$): N° of children, Minimum Dietary Diversity (Non-continued breastfeed), main water drinking source, n° of children < 5 years, having at least one intestinal/urogenital parasite, sickle cell, *P. falciparum*. Model adjustment: Pearson: $\chi2(40) = 27.3$, $p = 0.938$; Deviance: $\chi2(40) = 31.0$, $p = 0.845$; R2 Nagelkerke = 13.0% [4]. Variables excluded from the model ($p > 0.05$): Number of residents, latrine ownership, *P. falciparum*, Food frequency. Model adjustment: Pearson: $\chi2(176) = 170.5$, $p = 0.604$; Deviance: $\chi2(176) = 167.0$, $p = 0.675$; R2 Nagelkerke = 20.3%

having inflammation and only zinc deficiency and malarial inflammation stood significantly associated with Non-IDA (see Table 3). In the older age category, IDA was found to be associated age, stunting and inflammation, while children diagnosed with Non-IDA were more likely to have zinc deficiency, and inflammation without malaria.

Discussion

Prevalence of anaemia

In the present study, conducted in the Dande municipality in 2015, the prevalence of anaemia among under 5-year-old children was 44.4%, lower than previously reported for the Dande municipality (57%) [13]. We found that prevalence's were higher in children aged between 6-to-23 months (52%), comparatively to under 6 months and 24-to-36 months children (respectively 52, 36 and 35%). This is in accordance with children development [44, 45]. However, its contrary to national estimates reporting higher prevalence's in younger children (between 6-to-11 months, specially in 6–8 months children (reaching near 83%)), and worldwide estimates (reporting higher prevalence in 1-to-12 month children) [1, 11]. Nevertheless, the low density of under 6-month children should be taken into consideration.

The prevalence of IDA was also lower than expected (46% of all anaemic children), as it is generally assumed that half of the anaemia cases are due to iron deficiency [4, 46, 47]. This lower contribution of micronutrient deficiencies to the total anaemia, was also previously described in the South Sub-Saharan Africa (while an higher contribution of infections and sickle cell was estimated for the central and Western areas) [1, 4, 48]. Here, our results suggest that within this context, beside the factors compromising iron imbalance (such as blood loss, inadequate iron ingestion or compromised iron absorption), other associated factors may be of greater importance [4, 47]. Thus, this study add a modest contribution to the comprehensive work published by Kassebaum et al, by describing the factors specifically associated with the occurrence of IDA and Non-IDA in pre-school children of northern Angola, further discussed below [1, 4].

Factors associated with IDA and non-IDA

Here, adjusted multiple multinomial regression models showed that the relevant factors associated with the occurrence of IDA within this setting were (a) age (6-to-23-month children had 7.4 times more odds of having IDA than under 6 months children), (b) gender (males had 1.96 more odds than females) and (c) inflammation (particularly non-malarial inflammation). In the same models, the occurrence of Non-IDA was also associated with the children's age, besides zinc deficiency and overload, *P. falciparum* infection and sickle cell trait/anaemia (see Fig. 1).

The occurrence of total anaemia (in 2–15 years old children) have already been previously associated with gender, age, *P. falciparum* and *S. haematobium* infection in 2–15 years old children from this setting in 2010 [13]. Extending that knowledge, the present study documents that children's age associates differently with IDA and Non-IDA and that gender possibly influence more the occurrence of IDA. Those differential associations may be related to different underlying factors of IDA and Non-IDA within those groups [49]. For instance, the increased risk of IDA observed in the 6-to-23-month group may be potentially related with the higher iron requirements in children within these age group, as also reported in other African studies [48]. Regarding the differentiated influence of gender, it is suggested that males may have lower iron stores, and higher rates of iron deficiency than female infants [50, 51].

Our study also corroborates the relevant association between *P. falciparum* and anaemia, particularizing that in our study area is mainly associated with Non-IDA. Malarial anaemia (mainly severe anaemia) may result from acute and chronic haemolysis and/or systemic inflammation (that impair erythropoiesis), and considering that pre-existent iron deficiency is reported to worsen this condition, it would be expected that *P. falciparum* infections were also associated IDA [40, 48, 52–54]. Here, the higher frequency of malaria cases in the Non-IDA children could have contributed to the statistical significance observed and explain the higher analytical robustness. However, *P. falciparum* could also be less prevalent in the IDA group due to the lower availability of iron for parasite multiplication [55].

Besides the confirmation and knowledge extension of previously published results for this geographic area, we also document the relevance of infection-related inflammation as important factor for the occurrence of IDA anaemia, apart from malaria. Regarding the non-malarial parasites studied here, the literature mentions an "immune activation" effect mainly for *Schistosome* and *Giardia* infections [56–59]. Nevertheless, it should be considered that other infections, not studied here, could also be contributing to the occurrence of infection-related inflammation (such as HIV, tuberculosis and other tropical enteropathies), and consequently to anaemia [16, 52, 60].

One of the more important relevant evidence documented in this study is the association of Non-IDA with zinc levels, namely zinc deficiency associated with increasing IDA and zinc overload having a protective effect, In one hand, during zinc deficiency, the withdraw of zinc from tissues may occur, leading to increased hepcidin synthesis, which will reduce iron uptake, affecting

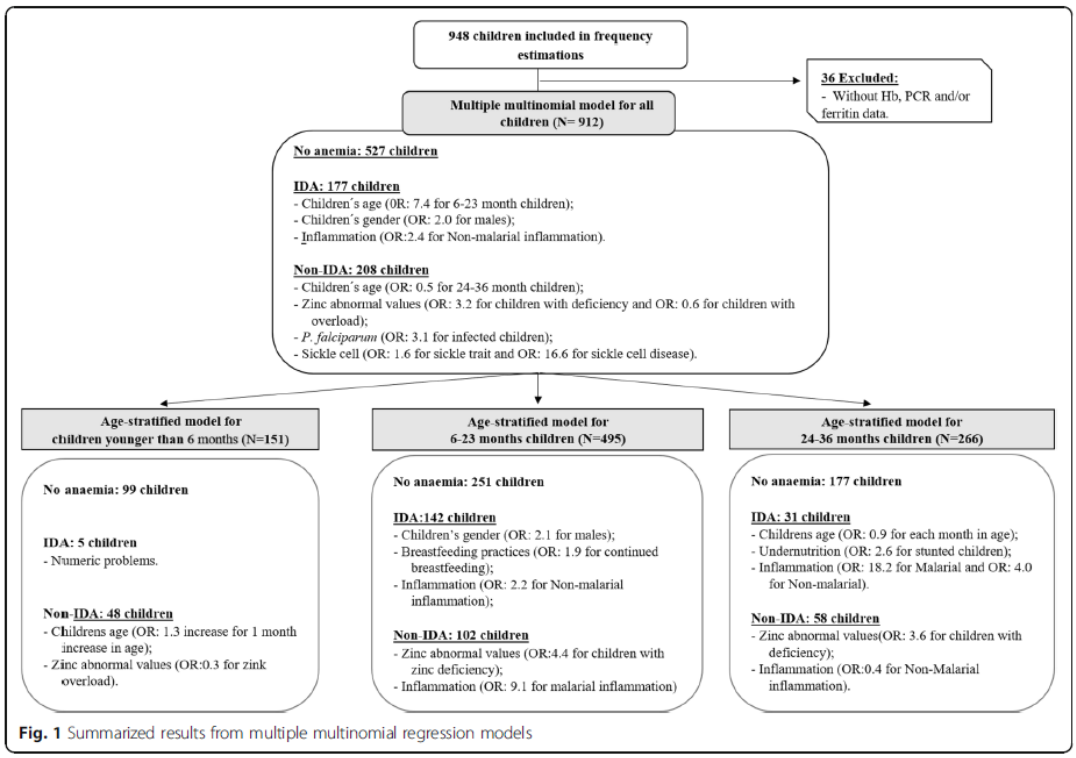

Fig. 1 Summarized results from multiple multinomial regression models

erythropoiesis, even in the presence of adequate iron stores [61, 62]. On the other hand, while zinc deficiency was reported to lead to immune dysfunctions (and consequently worse responses towards infections and increased infection-related anaemia), increased zinc levels may protect against enteric bacterial pathogens, possibly acting as an inhibitor of pathogen's virulence and preventing micronutrients malabsorption [63–65]. Thus, we hypothesized that zinc deficiency may be associating with iron status, inflammation and/or infections in the causality to Non-IDA. This kind of nutritional immunity could help explaining the protective (possibly confounded) effect of being infected with at least one intestinal/urogenital parasite observed on children with IDA in crude models, considering that the opposite association was expected [64, 66].

Lastly, the association between Non-IDA and sickle cell anaemia was not surprising, considering that this hereditary disease has been long known to present low average haemoglobin values (7–8 g/dL) [67, 68]. Newborn infants with sickle cell anaemia are reported to be healthy due to predominant production of fetal haemoglobin while in the uterus and neonatal period, but anaemia and haemolysis are evidenced after 4–6 months of age [68]. Also, the carriers of sickle cell trait (AS) were suggested to have a relative survival advantage over people with normal haemoglobin in regions where malaria is endemic, but this is neither absolute protection nor invulnerability to the disease [68, 69].

Age-related factors associated with IDA and non-IDA

In general, the proportion of anemia attributable to the nutritional, infectious and genetic causes discussed above may vary according to several physiologic and biologic aspects, as also according to the regional prevalence of anaemia etiologies and their underlying causes. Kassebaum et al., 2014, estimated that the anaemia cause-specific profile for children aging 0-to-27 days was composed mainly by IDA, hemoglobinopathies and infections (other than malaria, hookworms and schistosomiasis) [1, 4]. In children aging between 28 and 364 days, the contribution of IDA become less relevant, the impact of hemoglobinopathies is sustained and the contribution of Neglected Tropical Diseases and malaria become more relevant, shift that become more evident in 1

to 4 years old children [1]. The age-specific factors associated with IDA and Non-IDA are presented in Fig. 1.

Under 6 months children

Besides having age (monthly) variations within the under six-month children in the occurrence of Non-IDA, the statistically significant association between Non-IDA and zinc overload, discussed above, was sustained in this age group, suggesting that the protective effect of high levels of zinc may begin at early ages. Unfortunately, numeric problems prevented us from determining IDA associated factors in this age group.

6-to-23 months children

Here, children who had already been introduced to complementary food and were still breastfeeding, were more susceptible of having IDA than children that were in exclusive complementary feeding. Previously, Pasricha et al 2011 reported that Indian children that were continued breastfeed were more likely to receive poorer complementary fed, also belonging to highly food insecure households, and poorer micronutrient status [70] Despite that the breast milk is an important source of iron, its intake and absorption may be insufficient to meet the amount required for growth and complementary foods are expected to balance that [71, 72].

Also, our results regarding inflammation suggest that in this age group, non-malarial infections may be contributing more to IDA, while *P. falciparum* malaria may be contributing mainly to Non-IDA, both possibly through inflammation. Considering also the effect of zinc deficiency, inflammation and malaria on the occurrence of Non-IDA, the hypothesis of zinc playing an important role in the nutritional immunity of those children may become more plausible.

24-to-36 months children

At this age group, children with either non-malarial or malarial inflammation had more chances of having IDA, comparatively to children without inflammation. These observations may be in accordance with reports describing that the decreasing impact of IDA, and increasing contribution of malaria and Neglected Tropical Diseases (NTD) to the occurrence of anaemia, may be more relevant 1 to 4 years old children, when hookworm and schistosomiasis become important [4]. Besides this recurrent association with infections and/or inflammation, stunted children were observed to have more chances of having IDA, while children with zinc deficiency were more likely to have Non-IDA. Regarding stunting, it should be noted that nutritional anaemias, particularly IDA, are directly linked to micronutrient deficiencies (mainly iron deficiency), and possibly to the long periods of nutritional restriction that leads to stunting [15, 40].

Study strengths and limitations

Although some measures were applied to reduce bias and confounding, this study has associated limitations that should be considered when interpreting our results. Mainly, the small population sample could have limited the estimation of associations with diseases with low frequency, the occurrence of differential missing (which influenced the final denominators of composite variables) and the convenient sampling design of this study doesn't allow for result extrapolation to the Dande municipality. Also, this cross-sectional design may misrepresent close relations between predictors and intermediary steps in the causal pathway to anaemia. Furthermore, the lack of data of other relevant conditions/diseases that could lead to anaemia, such as other relevant infections (HIV), other enzymopathies (such as Pyruvate Kinase Deficiency), and other types of anaemia, such as acquired and hereditary aplastic anaemias, limit the complete comprehension of the problem. Also, some methodological constrains may have influenced the frequency estimation of intestinal and urogenital parasites studied here. Namely, impossibility to perform Kato Katz in diarrheal samples (limiting the diagnosis of helminths) and single sample diagnosis. For instance, it was reported that the Kato-Kats sensitivity to diagnose hookworms using only one stool sample, was 65.2% [73].

Conclusions

This study has observed that the main variables associated with IDA within this geographic setting are age, sex and inflammation, while the factors associated with non-IDA were age, zinc deficiency or overload, *P. falciparum* infection and sickle cell anaemia. While most of those associations were commonly reported for the occurrence of total anaemia in Africa, here they were associated in specific with IDA and/or Non-IDA. Additionally, the associations with inflammation, zinc deficiency and infections could be suggesting the occurrence of nutritional immunity in the pathway to anaemia within these Angolan children, calling for additional research. In age groups, zinc overload was suggested to protect under 6 months children from Non-IDA, while continued breastfeeding was associated with increased IDA prevalence in 6-to-23 months children, and stunting was suggested to increase the odds of IDA in 24-to-36 month children. This site-specific profile can inform the planification of preventive and corrective actions/programs.

Abbreviations

BMI: Body Mass Index; CISA: Health Research Center of Angola (Translated); CRP: C-reactive protein; G6PD: Glucose-6-phosfate dehydrogenase; HAZ: Height-for-age; Hb: Haemoglobin; HDSS: Health and Demographic Surveillance System; IDA: Iron deficiency anaemia; IYCF: Infant and Young Child Feeding; MDD: Minimum Dietary Diversity; Non-IDA: Non-iron deficiency anaemia; NTD: Neglected Tropical Diseases; PCR: Polymerase chain

reaction; WASH: Water sanitation and hygiene; WHO: World Health Organization; WAZ: Weight-for-age; WHZ: Weight-for-height

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Authors' contributions
CF - conceptualized the research question and participated in the design (mainly the laboratory operational procedures for parasitological, biochemical, and molecular analysis) and implementing the study (mainly coordinating and supervising the recruitment of participants in the field). Also, carried out the molecular analysis, performed the statistical analysis, drafted the initial manuscript, and revised the subsequent versions. AS - have adapted and structured the questionnaire, coordinated and supervised data collection in the field and critically reviewed the manuscript. JL - have designed the operational procedure for molecular analysis, provided technical support, and critically reviewed the manuscript. HB - helped conceptualizing the research question, critically oriented and supervised the initial statistical analysis and critically reviewed the manuscript. MB - helped conceptualizing the research question, participated in the overall study design and critically reviewed the manuscript. All authors approved the last version of the manuscript to be submitted and are responsible for this work.

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Availability of data and materials
Data supporting the results can be made available upon request.

Ethics approval and consent to participate
This study was approved by the Ethical Committee of the Ministry of Health of the Angola Republic. Children's caregivers (mainly the children's mothers) have signed an informed consent, after an information sheet was explained and delivered to them. Hospital-based and home-based consultations were held for the treatment of intestinal and urogenital parasites. Children with sickle cell were also followed in specific consultations. All the diagnostic and therapeutic resources used were provided free of charge.

Consent for publication
Not applicable.

Competing interests
The authors have no competing interests to declare.

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Publisher's Note

Paper III: Zinc deficiency interacts with intestinal/urogenital parasites to cause anemia in children under 5, Bengo – Angola

Zinc deficiency interacts with intestinal/urogenital parasites to cause anemia in preschool children, Bengo – Angola

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Abstract

Iron-unrelated anemia was associated with zinc pathological levels, while iron deficiency anemia was associated with inflammation. Herein we aim to investigate if the causal pathway between zinc deficiency and anemia is influenced (by nutrient deficiency, inflammation or infections). 852 children were enrolled in this transversal study, and crude multinomial models were fitted to search for associations and logistic regression models to investigate mediation effects and significance confirmed by Sobel test. 6.8% of children were found to have zinc deficiency, 19.4% zinc overload and 45.9% anemia. Children with high zinc levels were significantly less likely to be infected with *P. falciparum*, and children with zinc deficiency had 1.9 more chances of having non-malarial inflammation. Furthermore, we found: 1) no mediator or interactive effects among zinc deficiency and iron deficiency, 2) no significant evidences that inflammation could be mediating or interacting with

zinc deficiency to cause anemia and 3) no mediator, but a significant interaction effect between zinc deficiency and infections in the causal pathway to anemia (OR: 13.26, p= 0.022). This interaction appears to be stronger among children having iron deficiency anemia (OR: 46.66, p= 0.003). Multi-causal pathways are complex and other types of associations were not explored.

Keywords

Zinc, anemia, iron deficiency, infections, parasitic diseases, inflammation

Introduction

Zinc is an important trace element being involved in a large range of cellular processes, including cell structure synthesis, nucleic acid metabolism and membrane stabilization, while it participates in management of oxidative damage [40, 41]. It is a catalytic metal for about 300 enzymes and a structural metal for near 3000 proteins, also regulating enzyme activity [160]. Its homeostasis (the balance between intestinal absorption and excretion) was reported to be determined by dietary intake rather than zinc status [40, 160-162]. Recently, zinc was described to be a predictor of hemoglobin levels, and consequently associated with the occurrence of anemia, possibly contributing more to the development of anemia than iron itself [33, 38, 39]. There are 2 major mechanisms by which zinc could contribute to the occurrence of anemia that should be considered.

First, in the presence of iron deficiency, adaptation processes occur, corresponding to a tissue and/or systemic level responses [53]. Zinc may affect both processes, either by affecting local iron regulatory proteins (acting as a catalyst or affecting their expression) and/or by affecting the normal function of hepcidin (an iron regulatory hormone) [33, 38, 40, 53, 160, 163-166]. Furthermore, the depletion or overload of both zinc and iron can affect the expression of each other´s transporter´s and the expression of iron storage proteins [160]. Knezetal *et al* 2015, suggested that cellular zinc concentrations, along with intracellular iron levels could have a major role in the control and modulation of iron absorption, dictating the expression of proteins responsible for iron metabolism [33, 38, 160, 164, 166]. On the other hand, the regulation of hepcidin, a peptide hormone that have a strong influence on the intake of iron from diet, which leads to iron overload when present in low levels or to hypoferremia and anemia of inflammation when present in high concentrations, is also influenced by cellular concentrations of zinc (besides inflammation, infection, erythropoietic demand, hypoxia and body iron status) [160, 167-169]. Furthermore, during zinc deficiency, and in an effort to adjust to low zinc dietary intake, the system will withdraw zinc from tissues, leading to increased hepcidin synthesis

which will reduce iron uptake [160, 169]. Thus, poor zinc homeostatic adjustments, and a negative zinc balance could lead to iron being poorly absorbed (even within adequate iron stores) [160]. This reinforce the hypothesis that significant anemia might occur due to impaired mobilization of iron from diet, rather than due to inadequate dietary intake [160, 170].

Second, inflammation can also act as a regulatory factor in the association between zinc, hemoglobin and anemia. During inflammatory events, zinc is confined into cellular compartments decreasing the concentrations of plasmatic zinc in an attempt to withhold it from the pathogen [40]. This nutritional immunity was suggested to be mediated by inflammatory cytokines [41]. Furthermore, zinc it's among the trace elements whose metabolic profile change during both inflammation and infection [41]. Additionally, zinc deficiency was reported to lead to immune dysfunctions, that consequently lead to worse responses towards infections [41]. Furthermore, zinc deficiency was reported to account for near 18% of malaria cases, with zinc supplementation being associated with decreased prevalence of malaria [41, 42]. Clinical benefit of zinc supplementation has also been described in children with diarrhea, where decreased duration, severity/morbidity and occurrence of diarrhea were reported [41].

Despite the existence of plausible mechanisms for zinc-hemoglobin relations, there are limited *in vivo* data supporting them, emphasizing the importance of exploring mechanisms underlying those associations. Herein we aim to investigate if the causal pathway between zinc deficiency and anemia is influenced (through mediation or interaction) by nutrient deficiency (particularly iron deficiency), inflammation or infections. In specific, we aim at testing if iron deficiency, inflammation or infections influence directly the occurrence of anemia (or Non-Iron Deficiency Anemia - Non-IDA anemia) (hypothesis 1), or partially mediate the effect of zinc deficiency in the occurrence of total anemia (or Non-IDA) (hypothesis 2) or interact with zinc deficiency in its association with anemia (or Non-IDA) (hypothesis 3.

Material and methods

Study site and population

The present study is nested in a 12-months follow up efficacy trial, investigating the effect of nutrition and WASH/malaria educational community-based interventions in reducing anemia in preschool children from Bengo, Angola, approved by the Ethical committee of the Ministry of Health of the Republic of Angola [148]. The data analysed here was collected at the baseline moment from that

major study. In sum, it occurred in seven hamlets with functional health posts selected from the CISA´s study area [118, 119]. This area comprehends 4700km2, and near 60000 residents located within the Dande Municipality (70 km from the Capital Luanda), which are being monitored by the Dande Health and Demographic Surveillance System (HDSS) since 2009 [118, 119]. The hamlets were selected by convenience, based on the presence of functional peripheric health unit. After the explanation of the study, and that a verbal acceptance to participate was manifested, the field technician delivered a participant information form and a stool and urine containers to eligible families and instructed them to be present at the health post for evaluation. From all recruited families, 948 children attended the evaluation day at the health posts, an informed consent was signed (by the caretaker) for each one and children were evaluated. Only children with evaluable biochemical results for zinc pathologic levels were included.

Sample collection

Parasitological analysis comprised the diagnosis of *P. falciparum* malaria performed using a rapid diagnostic test (*SD BIOLINE* Malaria Ag *P.f*/*P.v*® (Standard Diagnostics, Inc., Republic of Korea) according to the manufacturer. Diagnosis of intestinal parasites were performed through fecal smear and centrifugal-sedimentation (using Kato-Katz technique and Parasitrap® kits (Biosepar, Germany)) and urogenital schistosomiasis by urine filtration, using Whatman® Nuclepore™ membranes, Merck, Germany) [138-140]. Biochemical analysis included determining blood levels of hemoglobin by imunocromatography using an Hemocue ® Hb 301 System (Angelholm, Sweden), serum levels of C-reactive protein, Ferritin and Zinc, respectively by turbidimetry, immunoturbidimetry and colorimetry (using an automated autoanalizer (BT1500) and CRP turbidimetric latex ®, Ferritin® and Zinc® kits from Quimica Clínica Aplicada S.A (Spain).

Statistical analysis

Total anemia was classified as hemoglobin levels (Hb) below 11.0 g/dL [4, 92, 144]. Furthermore, inflammation was classified as C-Reactive Protein (CRP) serum levels higher than 5 mg/L and children were considered to have Iron deficiency whenever serum ferritin was below 12µg/L (without inflammation), or below 30µg/L (with inflammation) [145]. The classification of Iron Deficiency Anamia resulted from the combination of the variables described previously, in sum Hb level below 11,0g/dL in the presence of iron deficiency. Pathological zinc levels were considered whenever, serum levels were bellow 70.0 µg/dL (Zinc deficiency) or above 150.0 µg/dL (Zinc overload) [146]. The prevalence´s of helminths and protozoa were determined as the proportion between infected children and those

delivering the correspondent sample. Children were considered to have diarrhea if caretakers reported that the children had 3 or more aqueous dejections per day in the last 2 weeks.

In this study, 95% confidence intervals (CI95) were estimated for the frequencies. Crude multinomial models were fitted, each with each independent variable and taking children with normal zinc serum levels as the reference category for the dependent variable (vs. Low zinc levels and high zinc levels). Logistic regression models were used to estimate the individual effects of independent variables and mediating (intermediate) variables in the occurrence of anemia. Mediation was considered to exist if: 1) the independent variable influenced significantly the mediating variable, 2) the independent variable influenced significantly the dependent variable and, 3) the addition of the mediating variable to the model altered significantly the effect of the independent variable in the dependent one [149, 150]. In addition to this method, Sobel test was used to determine the significance of the mediation effect, as proposed by Baron & Kenny (1986). Interaction is reported to occur when the effect of an explicative variable on the occurrence of anemia is different across the strata of another explicative variable [151, 152]. Here, an interaction was considered to exist if the combined effect of both variables were larger or smaller than the individual effects of A and B in the same model and statistical significance ($p = <0.05$) was observed.

Hypothesis under testing

Here we used an experimental-causal-chain design to investigate if zinc deficiency is mediating or is mediated by other variables with biologic/physiologic plausibility in the pathway to anemia. The relationships to be tested include the hypothesis that Inflammation or Parasites or Iron deficiency are directly associated with anemia (or Non-IDA) (hypothesis 1), or partially mediates the effect of zinc deficiency in the occurrence of total anemia (or Non-IDA) (hypothesis 2) or interact with zinc deficiency in its association with anemia (or Non-IDA) (hypothesis 3). Illustrations highlighting the relationships being experimented here are provided as supplemental material.

Results and discussion

Zinc pathologic levels and their associated factors

Among the enrolled 948 children in the main study, 852 had evaluable data for zinc serum levels. From those, 6.8% were found to have zinc deficiency, 19.4% had zinc overload and 45.9% had anemia. While children with zinc deficiency was found to have higher odds of having also Non-IDA (OR: 2.8, p-value= 0.001) and Non-malarial inflammation (OR: 1.9, p-value= 0.028), children with zinc

overload was found to have less chances of having Non-IDA (OR: 0.6, p-value= 0.030) and of being infected with *P. falciparum* (OR: 0.2, p-value= 0.015), when both groups were compared to children with normal zinc values (see table 1).

Table 1 – Frequency and associations between with pathological levels of zinc

	Frequencies among zinc pathological groups				**Association with zinc levels**				
	Total population	Normal	Low	High	**Normal**	**Low**		**High**	
	% (n/N)	% (n/N)	% (n/N)			OR (95% CI)	P-value	OR (95% CI)	P-value
Anemia									
No	54,1 (460/851)	53,5 (336/628)	36,2 (21/58)	62,4 (103/165)	Ref	Ref	Ref	Ref	Ref
Yes	45,9 (391/851)	46,5 (292/628)	63,8 (37/58)	37,6 (62/165)		**2,0 (1,2-3,5)**	**0,013**	**0,7 (0,5-1)**	**0,041**
IDA									
No	54,5 (460/844)	53,8 (336/624)	36,2 (21/58)	63,6 (103/162)	Ref	Ref	Ref	Ref	Ref
IDA	21 (177/844)	21,8 (136/624)	17,2 (10/58)	19,1 (31/162)		1,2 (0,5-2,6)	0,683	0,7 (0,5-1,2)	0,195
Non-IDA	24,5 (207/844)	24,4 (152/624)	46,6 (27/58)	17,3 (28/162)		**2,8 (1,6-5,2)**	**0,001**	**0,6 (0,4-1)**	**0,030**
ID									
No	61,8 (513/830)	61,2 (377/616)	70,7 (41/58)	60,9 (95/156)	Ref	Ref	Ref	Ref	Ref
Yes	38,2 (317/830)	38,8 (239/616)	29,3 (17/58)	39,1 (61/156)		0,7 (0,4-1,2)	0,157	1,0 (0,7-1,5)	0,945
P. falciparum									
No	94,7 (805/850)	93,3 (585/627)	98,3 (57/58)	98,8 (163/165)	Ref	Ref	Ref	Ref	Ref
Yes	5,3 (45/850)	6,7 (42/627)	1,7 (1/58)	1,2 (2/165)		0,2 (0-1,8)	0,168	**0,2 (0-0,7)**	**0,015**
At least one intestinal or urogenital parasite									
No	83,5 (628/752)	85,3 (464/544)	78,4 (40/58)	82,7 (124/150)	Ref	Ref	Ref	Ref	Ref
Yes	15,6 (117/752)	14,7 (80/544)	21,6 (11/58)	17,3 (26/150)		1,6 (0,8-3,2)	0,196	1,2 (0,7-2)	0,429
A. lumbricoides									
No	95 (678/714)	95,9 (493/514)	94 (47/58)	97,2 (138/142)	Ref	Ref	Ref	Ref	Ref
Yes	3,9 (28/714)	4,1 (21/514)	6 (3/58)	2,8 (4/142)		1,5 (0,4-5,2)	0,525	0,7 (0,2-2)	0,487
S. haematobium									
No	93,2 (478/513)	93,5 (348/372)	93,8 (30/32)	91,7 (100/109)	Ref	Ref	Ref	Ref	Ref
Yes	6,8 (35/513)	6,5 (24/372)	6,3 (2/32)	8,3 (9/109)		1 (0,2-4,3)	0,964	1,3 (0,6-2,9)	0,513
G. lamblia									
No	92,2 (647/702)	93,1 (475/510)	90 (45/50)	89,4 (127/142)	Ref	Ref	Ref	Ref	Ref
Yes	7,8 (55/702)	6,9 (35/510)	10 (5/50)	10,6 (15/142)		1,5 (0,6-4)	0,414	1,6 (0,8-3)	0,146
Inflammation									
No	54,3 (463/852)	54,5 (343/629)	41,4 (24/58)	58,2 (96/165)	Ref	Ref	Ref	Ref	Ref
Yes	45,7 (389/852)	45,5 (286/629)	58,6 (34/58)	41,8 (69/165)		1,7 (1-2,9)	0,057	0,9 (0,6-1,2)	0,401
Inflammation (									
No	54,8 (463/845)	55,1 (343/623)	41,4 (24/58)	58,5 (96/164)	Ref	Ref	Ref	Ref	Ref
Malarial	3,3 (28/845)	4,0 (25/623)	56,9 (33/58)	1,2 (2/164)		0.6 (0.07-4.4)	0.591	0.3 (0.07-1.2)	0.092
Non-malarial	41,9 (354/845)	40,9 (255/623)	1,7 (1/58)	40,2 (66/164)		**1.9 (1.1-3.2)**	**0.028**	0.9 (0.7-1.3)	0.664
Diarrhea									
No	59 (497/843)	59,5 (370/622)	71,9 (41/57)	52,4 (86/164)	Ref	Ref	Ref	Ref	Ref
Yes	41 (346/843)	40,5 (252/622)	28,1 (16/57)	47,6 (78/164)		0,6 (0,3-1)	0,069	1,3 (0,9-1,9)	0,104

Previously published results of this team described higher frequency of iron-unrelated anemia (Non-IDA) than Iron Deficiency Anemia (IDA) (22.8% versus 19.4%, respectively) [171]. Furthermore, in adjusted multinomial models, Non-IDA (but not IDA) was reported to be associated with zinc pathological levels (as also *P. falciparum* malaria and hemoglobinopaties), while IDA was reported to be consistently associated with inflammation (either combined or not with P. *falciparum* malaria) [171]. Thus, adding to this already established link between anemia and zinc levels in this population, the present study documented associations between pathologic levels of zinc, Non-malarial inflammation, and malaria.

In fact, zinc was described to be a predictor of hemoglobin levels, and consequently associated with the occurrence of anemia [33, 38, 39]. Several aspects were highlighted in the literature, relevant for this paper is the fact that zinc concentration could affect the absorption of iron (despite adequate iron stores) and could lead to IDA, that inflammation was highlighted as a regulatory factor in the association between zinc, hemoglobin and anemia (with zinc being confined into cellular compartments to decrease its availability to pathogen) and that zinc deficiency was reported to potentially lead to immune dysfunctions that consequently worsen responses towards parasites [33, 38, 40-42, 160, 164, 166]. While searching for evidences that support those plausible relations, we advise the readers to have in mind that these are complex multi-causal pathways, for whom not all variables were included and not all types of associations were explored (such as suppression or modered mediation).

Could zinc and Iron levels be contributing jointly to anemia?

Thus, the first biological plausibility investigated in this study is based on the assumption that under pathological serum levels (deficiency or overload) and a zinc imbalance, a poor iron absorption can occur, consequently contributing to the overall levels of IDA [160]. In our study, simple multinomial models showed associations of both pathological zinc status with anemia (OR: 2.0, p-value=0.013 and OR: 0.7, p-value=0.041, respectively for low and high zinc levels). However, when anemia was stratified by simultaneous presence/absence of Iron Deficiency, the association with zinc values were sustained only for Non-IDA. This is supported by the absence of associations between both zinc pathological levels and Iron Deficiency children that had normal hemoglobin values.

Additionally, when we investigated the hypothesis that zinc deficiency could be either mediating or interacting with iron deficiency in the pathway to total anemia or IDA, we found no statistical evidence that could corroborate these effects happening among the studied children. The contribution of other micronutrients and the type of relations with anemia and hemoglobin should be further investigated.

For instance, Shamim *et al*, have investigated the associations between zinc, vitamin B12 and alpha-tocopherol in pregnant woman, reporting positive association of those micronutrients with hemoglobin [172]. Additionally, Houghton *et al*, recently explored the relations between micronutrients and hemoglobin and anemia and found that zinc mediated the association of selenium and hemoglobin [38]. Nevertheless, from a point of view of anemia´s prevention those aspects are of high public health importance, and the lack of studies clarifying the type of those associations and documenting what it is occurring in vivo is of concern.

Could zinc levels and inflammation be contributing jointly to anemia?

In general, non-malarial parasites could cause anemia indirectly by leading to blunted intestinal villi (which lead to impaired micronutrient absorption), inflammation (which cause decreased erythropoiesis), chronic blood loss, and/or hemolysis, while malaria was reported to cause anemia by relating directly to hemolysis, but also by indirectly causing inflammation [32, 34, 112]. In a previous study published by Fançony *et al*, Non-malarial inflammation was reported to occur more frequently in children with IDA, than in children with normal hemoglobin (OR: 2.4, p-value<0.001), malarial inflammation showed no differentiated distribution between IDA, Non-IDA and Non-anemic groups, when all age groups were considered [171].Considering that there are reports describing that during inflammatory events, the concentrations of plasmatic zinc is decreased in an attempt to withhold it from the pathogen, and that this nutritional immunity could be contributing to anemia, we performed additional analysis to understand if zinc pathologic levels could be involved in those associations [40, 41]. We found that, despite that total inflammation had no statistically significant association with pathological zinc levels, children presenting zinc deficiency had 1.9 more chances of having non-malarial inflammation than children with normal levels of zinc, possibly corroborating the participation of zinc in the nutritional immunity to pathogens other than malaria.

Considering this, the second biological plausibility being tested here investigated the possibility that parasites and/or other pathological conditions could be causing inflammation, which could be mediating or interacting with zinc deficiency in the causal pathway to anemia. To further investigate if the pathway could have 2 levels, i.e, if inflammation was in turn related to parasites, having diarrhea or at least one intestinal or urogenital parasites, we also considered multiple associations in our mediating or interacting models (see figure S2 from the supplemental material). In the mediation model (postulating inflammation as a mediator variable), the effect of zinc deficiency on anemia remained similar to the direct effect (OR = 1.93, p = 0.022), indicating that the inflammation may not be mediating the effect of that relationship. This is also confirmed by the Sobel test (p> 0.05). This fact was also

observed when we removed children with IDA from the analysis (OR: 2.84, p = 0.001 to direct effect and OR: 2.80, p = 0.001 for indirect effect) or Non-malarial inflammation (OR: 1.38, p = 0.439 to direct effect and OR: 1.43, p = 0.387 for indirect effect) . Furthermore, we also haven't found significant evidences of interaction between zinc deficiency and inflammation in the causal pathway to anemia, as the odds of the combined effect (OR: 1.861, CI: 0.590-5.872, p=0.290) wasn't significantly different from their individual effect on anemia. Similar results were observed when the interaction was investigated only to Non-ID anemic children (OR: 1.787, CI: 0.528-6.049, p=0.351). When diarrhea and having at least one intestinal/urogenital parasite were included to the mediation or interaction models of zinc deficiency, inflammation and anemia the effects were still non-statistically significant. For instance, the odds ratio for multi-mediated between zinc deficiency, inflammation and diarrhea was 1.80 (95% CI: 1.02, 3.18, p=0.042), similar to the odds ratio for the direct effect (OR: 1.97 versus 95% CI: 1.12, 3.45, p=0.018) and, despite of being higher than the odds ratio of the direct effect, no statistical significance was observed for the odds ratio of the respective multi-interacted model (OR: 1.48, 0.786-2,801, p=0.223 for the direct effect and OR: 2.49 (0.58-10.798, P=0.221 for the indirect effect). On the other hand, the odds for the multi-interacted model was 5.44, (95% CI: 0.579-51.138, p=0,138), also higher than the direct effect (OR:1.81, 95% CI: 0.95-3.48, p=0.072) but not statistically significant. In sum, no relevant results were observed regarding the mentioned effects. Nevertheless, inflammation still being associated with anemia, either in children with concomitant *P. falciparum* infection or not, and thus should be further investigated.

Could zinc levels and infections be contributing jointly to anemia?

In this study, none of the studied intestinal/urogenital parasites were found to occur differentially among zinc groups, neither alone or in mixed infections. However, children with either low and high zinc levels were less likely of being infected with *P. falciparum* malaria, than children with normal zinc values (association that was significant only for high values of zinc). Thus, considering that zinc deficiency was reported to lead to immune dysfunctions that consequently worsen responses towards parasites, we hypothesize that zinc could be mediating or interacting directly with parasites to cause anemia (without the influence of inflammation) [41, 42]. Diarrhea was used as proxy for other enteric parasites. In the present study, no evidences of mediation relations, as hypothesized in the supplemental figure 1, were found. However, having at least one intestinal and/or urogenital parasites was found to interact with zinc deficiency in the causality of anemia (Table 2). The association was found to increase significantly the odds of IDA (OR: 46.66, p= 0.003). This could be in accordance with reports mentioning that the body also responds to pathogens by decreasing iron availability, further removing iron from circulation and storing it as ferritin [173]. This fact was reported to be

achieved through increased hepcidin, whose production is in turn influenced by pro-inflammatory cytokines [41, 174].

Table 2 - Results of the main effects investigated, occurring between zinc and the studied variables in the pathway to anemia.

Mediation		**Interaction**	
Effect tested: OR (95% CI), p-value		Effect tested: OR (95% CI), p-value	
Model 1: Iron Deficiency, Zinc deficiency and anemia (N = 673)			
Direct effect (ZD and anemia)	1.73 (1.26-2.36), p=0.001	Direct effect (Iron deficiency and anemia)	2.79, 1.42- 5.50, p=0.003
Mediated by ZD	1.77 (1.29-2.44), p< 0.001*	Interacted (ZD*ID)	0.39, 0.12-1.31, p=0.127
Model 2: Zinc deficiency, inflammation and anemia (N = 686)			
Direct effect (ZD and anemia)	2.03 (1.16-3.54), p = 0.013	Direct effect (Inflammation and anemia)	1.38 (0.60, 3.16), p=0.444
Mediated by inflammation	1.93 (1.10-3.38), p = 0.022*	Interacted (ZD*inflammation)	1.86 (0.5- 5.87), p=0.290
Model 3: Zinc deficiency, parasites and anemia ZD (N = 595)			
Direct effect (ZD and anemia)	2.30 (1.26-4.22), p=0.007	Direct effect (Parasites and anemia)	**1.57 (0.81-3.02), p=0.182**
Mediated by parasites	2.39 (1.30-4.40), p=0.005*	Interacted (ZD*parasites)	**13.26 (1.46-120.72), p=0.022**

* p-value (Sobel Test) > 0.05. ** p-value (Sobel Test) < 0.05. ZD – Zinc deficiency, ID – Iron Deficiency, Parasites: *S. haematobium*, *A. lumbricoides*, *T. trichiura*, *G. lamblia*, *H. nana*, *E. hystolitica* and *S. mansoni*.

Conclusion

In this study, we found that high zinc levels were found to protect against malaria whereas low levels increased the chances of malaria-unrelated inflammation. Furthermore, parasites were found to interact with zinc deficiency, significantly increasing the odds of either total anemia or IDA. No other single, mediation or interaction models studied here had relevant statistical significance. Nevertheless, these results should be interpreted with care, as multi causal pathways are complex and other types of associations were not explored, such as suppression or modered mediation. As far as we know there are no similar studies published in Angola, either highlighting the contribution of zinc deficiency to the occurrence of anemia and further exploring the nature of other associations of public health relevance.

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Author Contributions

Conceptualization, C.F, H.B and M.B; Methodology, C.F and M.B; Data Curation, C.F; Writing – Original Draft Preparation, C.F; Writing – Review & Editing, C.F, A.S, J.L, M.B and H.B; Supervision, M.B and H.B; Project Administration, M.B and H.B. All authors approved the last version of the manuscript to be submitted and are responsible for this work.

Conflicts of Interest

The authors have no conflict of interest to declare.

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Paper IV - Efficacy of Nutrition and WASH/Malaria Educational Community-Based Interventions in Reducing Anemia in Preschool Children from Bengo, Angola: Study Protocol of a Randomized Controlled Trial

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Protocol

Efficacy of Nutrition and WASH/Malaria Educational Community-Based Interventions in Reducing Anemia in Preschool Children from Bengo, Angola: Study Protocol of a Randomized Controlled Trial

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Abstract: Angola reports one of the highest infant mortality rates in the world, and anemia represents one of its important causes. Recent studies, in under-five children from the Bengo province of Angola, described high prevalence's, suggesting malaria, undernutrition and urogenital schistosomiasis as important contributors for the occurrence and spatial variations of anemia. Educational community-based interventions, either in Nutrition and Water, Sanitation, Hygiene and Malaria are recommended to correct anemia. Herein, we designed a cluster-randomized controlled trial to study the efficacy of two educational-plus-therapeutic interventions in the reduction of anemia: one in nutrition and the other in WASH/Malaria. Socioeconomic, nutritional, anthropometric, parasitological and biochemical data will be collected from all willing-to-participate children, aging under four and resident in the Health Research Center of Angola study area. Considering the multifactorial causes of this condition, determining the efficacy of both interventions might help documenting weaknesses and opportunities for planning integrated strategies to reduce anemia.

Keywords: Anemia; malnutrition; infections; educational interventions; nutrition; WASH/Malaria

1. Background

Anemia, especially severe anemia, is associated with increased child mortality rates [1]. Moreover, limitations in physical and cognitive development, leading to an increase in academic failure and susceptibility to infectious diseases, are also of great concern [1–4]. Regional studies conducted in the Dande municipality (Bengo province) reported a 57% prevalence of anemia in under-5 children, indicative of a severe public health problem [5]. In this setting, in addition to the expected association with undernutrition (responsible for nearly 13% of anemia cases), clusters of high risk of anemia were found to overlap with *Plasmodium falciparum* and *Schistosoma haematobium* risk zones (associated with 16% and 10% of the cases, respectively) [5,6].

Around half of anemia cases are estimated to result from Iron Deficiency (ID) [7]. However, in malaria endemic countries, routine iron supplementation remains controversial and is only undertaken with caution, as the practice has been associated with both an increased incidence

and severity of malaria, especially in non-iron-deficient anemic people [8–12]. Additionally, single nutrient deficiencies are rare, as insufficient ingestion of other hematopoietic micronutrients may occur and cause nutritional anemia. On the other hand, the high prevalence, intensity, reinfection and incidence rates of anemia-related infections can also contribute to iron deficiency anemia, potentially refractory to iron supplementation [2,5,13–16].

Integrating therapeutic (deworming) and preventive (either food-based or WASH/malaria education) strategies can simultaneously treat infections and increase hematopoietic micronutrient intake or reduce disease transmission, which could result in the reduction of malnutrition and anemia [2,14,17–32]. Several comprehensive intervention strategies have correspondingly been tested. However, they frequently evaluate the impact of education on nutrition or on infectious etiologies (whether or not combined with drug therapy) separately [17–29,31]. Thus, differences in their designs and methodologies hinder the comparison and evaluation of results, rendering it difficult to define the best approach to reduce anemia. For instance, differences on the target population (adult or children), dimensions of the learning package (e.g., for sanitation: stool disposal, water quality or supply), the delivers (community promoters/volunteers, teachers, local health workers, community leaders), the place where education occurs (at health center or at the communities), the number of intervention contacts, type of contacts (group meetings and/or individual contacts), duration of the intervention/observation and mainly the combination with other strategies (micronutrient supplementation, construction of latrines and hand-washing mechanisms, etc.) can be observed [17–29,31]. Furthermore, one of the most inclusive studies was published recently by Humphrey et al. 2019, and investigated the effect of two WASH interventions, either alone or combined with improved Infant and Young Child Feeding (IYCF), concluding that education in nutrition reduce anemia and stunting, but adding WASH to the intervention had no major improvements on those effects [33]. Similar results were reported by Null et al. 2018 within a cluster-randomized trial using interventive groups with several WASH and nutrition combinations [34]. Neither study investigated the effect of combining deworming, nor was the impact of malaria preventive actions included, and as far as we know, there are no published protocols or results of such studies implemented in Angola.

1.1. Aim

This paper describes the design of a cluster-randomized controlled trial that aims to compare the efficacy of two complex, community-based interventions: (1) nutritional education in the reduction of nutritional anemia, and (2) WASH/malaria education in the reduction of anemia caused by infection, both combined with a test-and-treat therapeutic approach.

1.2. Hypotheses under Study

(1) The nutrition arm assumes that the test-and-treat approach will clear infections and that education in nutrition will improve the knowledge and awareness of caretakers as regards the causes, prevention and treatment of nutritional anemia, which in turn may result in behavioral changes that reduce the prevalence of inadequate breastfeeding and IYCF. This could in turn improve dietary diversity, by increasing the consumption of erythropoietic-rich foods (iron, vitamin B12, vitamin A and folate) and enhancers of iron absorption (vitamin C), and decreasing the inadequate consumption of iron absorption inhibitors (polyphenols, phytates, calcium, etcetera)) [24,35,36], leading ultimately to a decrease in the prevalence of nutritional anemia.

(2) The WASH/malaria arm assumes both that the test-and-treat approach clears infections, and that education in appropriate water, sanitation, personal hygiene and malaria prevention practices improves the knowledge of caretakers regarding the causes and preventive measures against infections, which in turn would lead to behavioral changes, and consequently to reduced prevalence, intensity, incidence and re-infection rates of those diseases [31,37–40]. Thus, considering that parasitic infections are important causes of anemia within this setting, this intervention would consequently decrease the occurrence of infection-related anemia.

In our study, the intervention in the control group targets the treatment of infections, the intervention in the nutrition arm targets nutritional anemia and nutrition-related causes, and the WASH/malaria intervention focuses on infections and infectious-related anemia. Furthermore, it should be considered that non-malarial infections can also cause anemia through nutritional deficiencies, by causing blunted intestinal villi (which lead to impaired micronutrient absorption) [41]. Thus, by trying to prevent anemia through these 3 possible pathways, we can simultaneously investigate (1) whether combining education in nutrition with a test-and-treat approach could reduce nutritional anemia more than only treating intestinal/urogenital and malaria infections; (2) whether combining education in WASH/Malaria with a test-and-treat approach could reduce nutritional anemia more than only treating intestinal/urogenital and malaria infections; and 3) which of the educational interventions can prevent nutritional anemia the most.

2. Materials and Methods

2.1. Study Setting

This study targets hamlets with functional primary health care facilities within CISA's Dande HDSS study area [42]. The surveyed area is located in the Bengo Province (Dande municipality), and includes all the hamlets in the communes of Úcua, Caxito, Mabubas, as well as some hamlets in Kikabo and Barra do Dande [42]. Within this area, between 2010 and 2014, the total resident population varied between 63,081 and 58,645, with the percentage of under-5 children ranging from 17.7% to 13.9%. Additionally, the fertility rate varied between 4.8 and 3.7 births per woman, with the crude birth rate ranging from 40.4 to 24.2 live births/year/1000 people and the under-5 mortality rate fluctuating between 92.1 and 60.1 per 1000 live births [42]. As the entire population of Kikabo and Barra do Dande are not fully surveyed by the Dande HDSS, they will not be included in this study. Although 15 hamlets with health facilities are located within this area, only those providing daily primary care practices will be included in this study [43].

2.2. Study Design

Baseline Assessment (Pre-Intervention Evaluation)

There were two qualitative pilot studies: (1) to characterize mother-to-child nutritional practices (as a basis to structure the questionnaire) and (2) to design a locally adequate Food Photography Atlas (FPA) for children U5 (for the 24 h recall evaluation). The first study was conducted between August and September of 2014 and enrolled 808 children aging 0–59 months and their caretakers in a two-stage cluster sampling strategy. Using a Portuguese translated questionnaire, adapted from ProPAN 14, we collected data on children's breastfeeding, feeding practices and detailed food consumption in the previous 24 h [44]. As a result, data regarding the commonly consumed food was collected and a preliminary FPA S was produced. Following this, the second study, aiming at validating the previously mentioned FPA and to allow for the collection of age and context-specific food portion and nutrient ingestion among preschool children from the Bengo province, was conducted between February and March 2015. This was a convenient cross-sectional study that enrolled 75 primary caretaker residents in the Mabungo and Riceno hamlets within CISA's study area. In sum, dishes were prepared according to local recipes, food photographs were produced, tested and selected, portion sizes were estimated and validated, and the reproducibility of this tool was evaluated. These results are being published elsewhere.

Using these tools, a pre-intervention evaluation, aiming to characterize the study population regarding anemia and its etiologic profile (by specific age groups (children aged under 6 months, between 6 and 23 months and between 24 and 36 months), will be performed.

2.3. Participants

All under-4 children and their mothers/caretakers, resident in the selected hamlets, will be considered eligible and invited to participate. This age group was selected because children are

expected to have limited mobility, to receive higher parental attention and to have their exposure to contaminated environments more controllable than school aged children. Children will be subject to exclusion whenever having recently received blood transfusions, report adverse reactions to albendazole and/or to praziquantel, and when their caretakers do not commit to completing both the evaluation and the educational moments of the study. Only children with pre-intervention evaluation will be allocated to the Randomized Controlled Trial (RCT).

2.4. Training for Evaluation Moments

At the beginning of the study, 6 field workers and 2 nursing technicians will be selected from the study area and trained. The one-week training will incorporate: (1) an introduction to the research questions, goals and study design; (2) basic concepts regarding the diseases studied; (3) methodologies for data collection (structured interviews, anthropometric evaluations, recognition of signs and symptoms of micronutrient deficiency, measurement of temperature); and (4) communication skills and the collection/delivery of information. Additionally, nursing technicians will undergo 3-day training on: (1) the treatment and referral protocol adopted in this study; (2) best practices for drug administration in young children; and (3) the management of domiciliary and health unit treatments. Blood, urine and stool samples will be collected before their processing by experienced CISA resident laboratory technicians. A field work simulation class will be conducted with all personnel involved in this survey at the end of all training sessions.

2.5. Recruitment of Participants

A house-to-house recruitment team will identify the children eligible in each hamlet, explaining the importance of the study both to local authorities and caretakers, delivering stool and urine containers and explaining the best practices for sample collection. This team will recruit up to 45 children per day, and orient them to attend the next evaluation at the hamlet health post. Those evaluations will be carried out in each hamlet until all eligible willing-to-participate families have been notified.

2.6. Baseline Data Collection and Sample Storage

A structured questionnaire is to be administered to mothers or other primary caretakers to collect information on identification, breastfeeding, complementary feeding practices and health care practices, supplementation and vaccination history, WASH and malaria practices. Weight and height, collected using electronic or platform pediatric scales and measuring harness or adult stadiometer, will be used to calculate anthropometric indices for the diagnosis of undernutrition in children and mothers according to WHO standards [45,46]. Peripheral blood will be collected in accordance with WHO good phlebotomy practices [47]. For biochemical analyses, blood will be collected in 1.1ml Z-Gel microvette tubes, centrifuged for the separation of the serum and stored at −20 °C and blood for molecular analysis will be stored in air-dried filter paper until processing. Mothers/caretakers will be instructed to collect a stool sample on the day before evaluation and to collect urine in the morning of evaluation day. Urine samples are to be conserved in formalin (10%) to prevent larvae eclosion.

2.7. Sample Processing

Stool, urine and blood will be analyzed parasitologically. Blood protozoa (*Plasmodium falciparum* and *Plasmodium vivax*) will be determined by the Rapid diagnostic test (*SD BIOLINE* Malaria Ag P.f/P.v (HRP-2/pLDH, Standard Diagnostics, INC, Suwon City, Korea)), intestinal helminths (*Ascaris lumbricoides, Trichuris trichiura, Hymenolepis nana* and *Strongyloide stercoralis*) determined through fecal smear according to the Katokatz technique, intestinal protozoa (*Giardia lamblia* and *Entamoeba hystolitica/dispar*) evaluated through fecal centrifugal-sedimentation (using Parasitrap®System, Biosepar, Mühldorf, Germany) and urogenital helminth (*Schistosoma haematobium*) determined by urine filtration [48,49]. Biochemical analyses will comprise quantifying hemoglobin directly from peripheral blood (using Hemocue ®Hb 301 System, Angelholm, Sweden), serum ferritin, C-reactive protein and

zinc through kits from Quimica Clínica Aplicada (Quimica Clínica Aplicada S.A., Tarragona, Spain) and an automated analyzer (BT1500, Biotecnica Instruments S.p.A, Rome, Italy), serum retinol through high-performance liquid chromatography with a protodiode-array detection, and vitamin B12 and folic acid by chemiluminescence immunoassay (using VITROS®Immunodiagnostic kits, Ortho Clinical Diagnostics, Inc. Bridgend, UK) [50–53]. Molecular analysis will include DNA extraction (by Chelex method), screening for sickle cell trait and disease (by PCR) and G6PD deficiency (by rtPCR) [54,55]. Malaria diagnosis will take place on site, and stool/urine samples will be analyzed within 24/48 h of collection (depending on the day of the week). The laboratory analyses will be performed by trained technicians and 25% of tests subject to confirmation by experienced laboratory supervisors.

2.8. Drug Therapy

Children diagnosed with *Plasmodium falciparum* malaria, urogenital schistosomiasis and/or intestinal parasites, will receive treatment, respectively with artemether/lumefantrine (20/120, plus paracetamol (15 mg/kg/dose for temperatures above 38 °C)), albendazol (400 mg/single dose for *Ascaris lumbricoides*, *Trichuris trichiura* and hookworms) and praziquantel (40 mg/kg for *Schistosoma haematobium* and *Schistosoma mansoni* (splited into two intakes, 6 h apart) and 25 mg/kg for *Hymenolepis nana*), according to the national therapeutic guidelines and specialist bibliography [56–59]. *Plasmodium falciparum*-infected children are to be treated on the evaluation site (unless signs of severe malaria are observed) and all albendazole treatments (except for children aged younger than one) will be administered in the household by nurse technicians. The remaining infected children will be treated at specific consultations with CISA's pediatrician at the health centers. Furthermore, any children diagnosed with sickle cell disease will be referred to the Anemia Patient Follow-up Consultation, held at the Bengo General Hospital. Drugs will be provided by CISA through the entire duration of the study.

3. Cluster-Randomized Controlled Trial

3.1. Sampling Strategy

In this study, the sampling strategy chosen was a non-probabilistic (convenient) sampling. In sum, we aimed to include (through a census approach) all eligible children (under the age of 4) resident in administratively and geographically isolated hamlets with functional health posts, in turn located within the CISA's HDSS study area (considered as cluster units). We chose hamlets with functional health posts due to the greater facility in mobilizing the population and corresponding logistical advantages. On the other hand, the census approach (within those clusters) was adopted because variations in the density of eligible children, estimated according to data from 24-10-2014 extracted from CISA's HDSS database, was expected, and the real density in each cluster was needed.

3.2. Randomization

The hamlets will be considered as cluster units. For randomization, the names of the hamlets will be written down on pieces of paper and placed in a bag and then successively removed. The first two papers to be removed will be attributed to the Nutrition arm, the following two to the WASH/Malaria arm and the next pair to the control with this procedure repeated until there are no papers left in the bag. This process will be carried out by the two CISA researchers coordinating the study.

3.3. Blinding

This will be an unblinded study.

3.4. Training of Providers

Field and nursing technicians involved in the evaluation (baseline and follow up waves) will be assigned to the different intervention arms, being responsible for the counselling sessions (monitoring, encouraging and continuously promoting behavioral change), under the direction of a supervisor

Their previous training will be reinforced with theoretical and practical sessions on (1) the core aspects of the target disease and conditions (specifically, "What is the disease/condition", "What are the signs and symptoms", "What is causing them", "What can mothers do to prevent them"), (2) counselling techniques (evaluating the emotional state of the caretaker and adapting counselling techniques to the emotional state), (3) household environment and risk behavior evaluation, according to the respective interventional arm they belong to. Within each intervention arm, they will perform one cycle of three-monthly visits to a group of households before then moving onto another group of families.

Following the first training stage, refresher sessions will occur before the follow up evaluation moments and before every domiciliary personalized counselling. At the beginning and at the end of each training stage, technicians will undertake a written evaluation test and a score will be generated. Additionally, 20% of all counselling sessions (per technician) will be subject to evaluation by a supervisor, who will similarly produce a counselling score for each trainee.

3.5. Training of Receivers

The receivers, i.e., the targets for the behavior change interventions, will be mothers and/or primary caretakers of the children studied. After the baseline assessment, the mothers in each intervention arms will be trained according to the respective "targets within the educational package" described in Table 1. The objective is to empower mothers with knowledge of basic principles regarding: (1) anemia and its nutrition-related etiologies and (2) anemia and its infections-related etiologies (in particular, what are the signs and symptoms? How did the child become at risk? How can mothers prevent this and what they should do whenever their children fall sick?) and to stimulate inadequate mother-to-child behavior changes. Technicians will document pre-and post-intervention alterations in the household environment and mother-to-child practices (according to the process indicators described in Table 2).

Table 1. Summary of the treatment given in each arm.

Type of Action	Nutrition Arm	WASH Arm	Control Arm
Diagnosis and treatment of: - Malaria (artemether-Lumefantrine 20/120), - Schistosomiasis (Praziquantel) - Intestinal parasites (Albendazol and Tinidazol) Implemented at the baseline and then every 6 months until the end of the study.	Provided	Provided	Provided
Distribution of bednets at the baseline	Provided	Provided	Provided
Distribution of soap and lye at the first and second follow ups	Provided	Provided	Provided
Personalized, home-based counselling of primary caretakers	Provided	Provided	Not provided
Targets within the educational package Each visit will address 2 behaviors of the specified targets topics, moving to the next pair in later visits	(1) Breastfeeding; (2) Complementary feeding *; (3) Weekly adequate food diversity; (4) Appropriate number of meals; (5) Adequate amount of food; (6) Responsible feeding; (7) Disease and alimentation; (8) Hygiene and food safety.	(1) Bednet usage; (2) Reducing mosquito breeding sites (3) Prevention of open sky defecation; (4) Latrine cleaning; (5) Adequate hand washing; (6) Healthy backyard environment; (7) Adequate water availability, treatment, transportation and storage; (8) Adequate personal hygiene.	Not applicable
Number of counselling visits	6	6	Not applicable
Activities within the community groups	(1) Discussion of nutritional value of foods, the effect of processing and the most common foodborne parasites; (2) Demonstration of fish-and-meat dishes; (3) Demonstration of combinations for breakfast and lunch; (4) Taste and score of recipes; (5) Distribution of illustrative notebook with recipes.	(1) Identification of focus of Soil-transmitted helminth (STH) contamination; (2) Identification of children's risk behavior in the community; (3) Identification of household indoor resting mosquitos and outdoor breeding sites; (4) Construction of household washing hand mechanisms; (5) Correct montage of bednet; (6) Children hand-washing classes.	Not provided
Number of community group sessions	6	6	Not applicable
Total duration of the follow up	12 months	12 months	12 months

3.6. Recruitment and Promotion of Adherence

To relocate families, field technicians will perform a house-to-house invitation strategy, using Dande's HDSS household identification system, reference points and family phone numbers. To increase adherence and promote participant retention, the hamlet coordinators and/or traditional authorities will be invited to be present at all phases of the study. Furthermore, incentive kits will be distributed to all children during the follow up moments, namely a nutritional kit (water and yogurt) and a WASH/Malaria kit (soap and lye).

3.7. Educational Package for Receivers

Families/caretakers allocated to the interventions are to receive six personalized counselling sessions at their homes and will be invited to participate in six community workshops for practice lessons. The overall treatment given in each arm, throughout the duration is described in Table 1.

Illustrative materials, adapted from UNICEF, will be used for these counselling sessions, as well as posters highlighting key health messages [60,61].

The practical cooking lessons within the nutrition study arm will aim at reinforcing the adoption of a diet with a Minimum Dietary Diversity and Minimum Meal Frequency and at increasing iron, folic acid, vitamin A- and B12-rich food ingestion [62]. Accordingly, after evaluating the ingredients available at the local market, aliments within the food groups of (1) grains, roots and tubers, (2) legumes and nuts, (3) dairy products, (4) flesh foods, (5) eggs, (6) vitamin A-rich fruits and vegetables, and (7) other fruits and vegetables will be identified, and easy, affordable and pleasant-to-the-palate recipes will be developed. Within those groups, food sources of heme-iron (red meat and poultry) and non-heme iron (specifically green leaves), will receive major attention [63–67]. Data collected at the qualitative study mentioned above, aiming at assessing the main feeding practices of infants and young children, will be used to assist this purpose. Classes will comprehend: (1) discussing the nutritional value of local foods, the effect of processing them on their nutrient value, (2) identifying the most common foodborne parasites, (3) demonstrating the fish-and-meat dishes to be used in the meals, (4) demonstrating combinations to be used at breakfast and lunch, (5) tasting and scoring recipes and (6) distributing illustrative recipe notebooks for practice.

The practical sessions within the WASH/malaria intervention arm will aim at preventing malaria, schistosomiasis and STH in children, particularly decreasing the transmission rate and consequently lowering their incidence and reinfection rates. Activities within the community groups will include: (1) identifying local points of possible STH contamination and children's risk behaviors, (2) visiting target households to identify mosquito indoor resting and outdoor breeding sites, (3) constructing household washing-hand mechanisms, (4) correctly mounting of bed-nets and (5) running child hand-washing classes.

Table 2. Indicators of impact, provision, utilization and coverage being collected in this study.

Impact Evaluation	Process Evaluation		
	Provision * Indicators	Utilization Indicators	Coverage * Indicators
1. Primary outcomes 1.1 Impact on health - Variation of hemoglobin levels; - Anemia prevalence reduction. 2. Secondary outcomes 2.1 Impact on Health - Iron deficiency anaemia reduction; - Micronutrient deficiencies (iron, folate, vitamins A and B12, zinc and vitamin E serum levels); - Prevalence reduction, intensity, incidence and reinfections rates of STH, *P falciparum* and *S. haematobium*; - Prevalence reduction of Stunting (height-for-age) and wasting (weight-for-height). 2.2 Impact on Behavior - Exclusive breastfeeding (0–5 months) and continued breastfeeding (12–15 months) [68]; - Increase of Minimum dietary diversity prevalence (6–23 months) [68]; - Increase of Minimum Meal Frequency prevalence [68]; - Increase of iron and vitamin A rich foods consumption; - Reduction of morbidity from diarrhea (reported).	1. Acquired knowledge - Theoretical test scores (evaluation of knowledge); - Practical test scores (evaluation of counseling performance); - Self-counseling efficacy evaluation. 2. Counseling performance - Number of families dropping outs from evaluation moments per technician; - Number of families successfully visited for counseling by each technician; - Number of notified participants present in each community groups per technician; - Number of scheduled visits held successfully per technician.	1. Acquired knowledge of receivers -Theoretical test scores; - Practical test scores (participation in community groups); - Self-evaluation at mother-to-child care. 2. Participatory level of receivers (adherence) - N° of attendances to evaluation moments; - N° of scheduled visits (both in person and by telephone call) held successfully; - N° of scheduled visits that had to be redials due to absence or unavailability; - Mood state of the receiver between visits; - N° of attendances to communitarian group. 3. Household environment evaluation (observation) - N° of household hand washing mechanisms; - N° of communitarian latrines around the house; - Cleaning conditions classification of household latrines (if existent); - Cleaning conditions classification of the backyard; - Presence of pets loose in the yard and stool. 4. Mother-to-children care practices - Children using bednets; - Children sidewalks; - Children with clean nails.	1. Services provided at evaluation moments - N° of hospital-based consultation held for parasite treatment; - N° of domiciliary-based consultation held for parasite treatment; - N° of hospital-based consultation held for sickle-cell follow up; - N° of children referred to the emergencies with severe anemia, severe malnutrition and non-malarial fever who reported to have been followed. 2.Participatory level of receivers (adherence) - Proportion of child-caretaker pair present in all evaluation moments; - Proportion of child-caretaker pair presenting in all counseling moments; - Proportion of caretakers participating in all community groups; - N° of notified child-mother pair failing the hospital-based consultations for parasite treatment or sickle-cell follow up; - N° of child-mother pair failing to picking up the sickle-cell drugs.

* Provision refers to the intervention provided by technicians and utilization refers to the action expected from receivers when provision is delivered.

4. Data Collection

Educational Interventions

Caretakers participating in the intervention arms will have a monitoring file (consisting of six structured questionnaires (one for each monthly visit), applied by the technician to document behavioral alterations at every counselling visit and to record attendance to the community group sessions. Those educational interventions will be intercalated with cross-sectional evaluations. Those moments will have the same design as the baseline assessment, occurring respectively six and twelve months after the beginning of the study.

Data on the primary and secondary outcomes, which will be collected only in the follow up moments, aim at evaluating the impact of interventions. Considering that interventions are expected to influence either health and behavioral aspects, impact indicators are expected to provide information on changes in their frequency, and potential associations. On the other hand, data on the process indicators, which will be collected throughout the study, aim at assisting the evaluation of the quality and success of intervention's delivery process. Thus, data regarding the provision, utilization and coverage aspects of the delivery process are expected to facilitate attributing the changes in primary and secondary outcomes to the intervention, by demonstrating that the intervention was of sufficient magnitude and occurred in a temporal sequence. Specifically, (1) provision indicators are expected to inform about the quality of provider's training and performance, and if the requirements for provider's optimal performance was delivered to them; (2) utilization indicators are expected to inform about the quality of receiver's sensibilization, their receptivity to interventional activities, acquired knowledge and alterations of inadequate behaviors toward children; and (3) coverage indicators aim at providing information regarding the quality of the interventional activities, such as the adherence to them.

5. Statistical Analysis

5.1. Data Management

Questionnaire data, both from the three evaluation moments and from the six educational visits, will be double entered by CISA's data loggers into digital forms forming an Open Data Kit (ODK) aggregate server. Afterwards, these files will be converted into Excel sheets and exported to SPSS for statistical analysis. Anthropometric indices will be extracted from the Excel files produced and entered into a WHO Anthro software (version 3.2.2, WHO, Geneva, Switzerland) to calculate z-scores and classify malnutrition. The laboratorial data will also be similarly entered into Excel®(Microsoft Corporation, Redmond, Washington, DC, USA) files and exported to IBM SPSS software, version 24 (IBM Corp, Armonk, NY, USA) for statistical analysis.

5.2. Baseline Assessment Evaluations

The nutritional quality of children's diets (24–36 months) will be determined in accordance with the Children's Individual Dietary Diversity Score Indicator (IDDS) [69,70]. The prevalence of malnutrition in children will be calculated by WHO Anthro software (version 3.2.2). The chi-square test will be used to compare proportions, in addition to calculating the attributable fractions.

Pre-and post-intervention and intervention versus control arms evaluations.

Primary and secondary impact indicators will be evaluated between the baseline and the follow ups (for each study sample), and between the intervention and control arms. Outcomes will be compared analyzing for variance in the continuous variables and logistical regression for the categorical variables. Adequacy and plausibility arguments will be used, by analyzing the process indicators (described in Table 2) in accordance with the conceptual maps. Relative risk will be calculated in order to compare the cumulative incidence of anemia among the intervention and control arms.

6. Discussion

The efficacy of interventions to reduce anemia can comprise both educational and therapeutic dimensions [2].

Deworming plus nutrition education has been reported to reduce anemia from 82.0% to 55.4%, while increasing the consumption of leafy green vegetables from 44.7% to 60.6% [24]. Furthermore, exclusive educational interventions targeting nutrition improvement, increased the ability of mothers to identify malnutrition (from 15% to 99%), increased exclusive breastfeeding (79% versus 48% in the control group), translated into weight gain (0.86kg versus 0.77kg in the control group), increased vegetable feeding, nutrient-dense foods at lunch (11% difference between the intervention and control groups), dietary requirements for energy, iron and zinc, complementary feeding, and significantly reduced rates of stunting [17–21,35,71].

Exclusive WASH educational interventions, health education on soil-transmitted helminths and schistosomiasis, significantly increased knowledge, reduced both the prevalence of *Ascaris lumbricoides* and *Schistosoma mansoni* and the incidence of hookworms and the intensity of trichuriasis, ascaridiasis and hookworms [25,26]. When deworming is added to WASH education, the prevalence and intensity of STH decrease and school children scored higher than the control on STH knowledge [26,31]. Studies evaluating educational approaches to prevent malaria report improved knowledge regarding breeding sites, bednet use and indoor spraying, increased windows and door net usage and alongside maintaining clean environment practices and leading to a reduced number of reported malaria episodes and the incidence of fever [27,28,39,72].

There are reports of intensity reduction (for *Schistosoma haematobium, Ascaris lumbricoides, Trichuris trichiura* and hookworm) and variable cure rates when participants are exposed only to deworming with PZQ or ALB. However, re-infections can occur rapidly [73–82]. While some authors report that the link between exclusive drug therapy and hemoglobin increase remains inconclusive, others report that exclusively deworming (with ALB and PZQ) may increase mean hemoglobin [83,84]. Additionally, treating malaria has an important impact on anemia reduction [85].

The studies investigating different combinations of those interventions present considerable differences in their designs and methodologies, making it hard to value and compare results [17–29,31,83,84,86–88]. Despite those results, only a few studies have simultaneously implemented a therapeutic plus educational mother-to-child nutrition intervention and compared it with a therapeutic plus educational mother-to-child WASH/Malaria practices [89].

Evaluating the efficacy of educational interventions represents a great challenge, as they are complex, have long causal pathways between the intervention and the outcome, span several intermediate levels and interacting components (in turn susceptible of modifying the effect under measurement and both the internal and external validity of the findings) [90]. One relevant dimension of this complexity is the number of target groups and the number of behaviors required by either those delivering or receiving the interventions for the impact on the outcomes to occur. Educational interventions depend on the knowledge acquired from field technicians, their performance in transmitting that knowledge, skills to conduct community activities and to monitor mother/caretaker practices. Additionally, mothers are expected to be receptive to counselling, gaining awareness of correct parental practices and changing inadequate behaviors and practices. In this study, we postulate that, for a single population, evaluation design, indicators, therapeutic approach and timeline, we will be able to more consistently and realistically describe and compare the effect of: 1) a therapeutic plus educational nutrition; and 2) a therapeutic plus WASH/Malaria interventions on the occurrence of anemia.

Nevertheless, considering the complexity of the design proposed, we can anticipate some difficulties. Randomization may not grant the elimination of all non-measured confounding factors just as the prevalence of several outcomes may be difficult to dilute within the clusters [91]. Additionally, there is also the possibility of poor compliance and differences in the intervention dosage delivered and the amount of response produced. Here, we will distribute nutrition and WASH/Malaria

kits during the evaluation moments to prevent low adherence. Furthermore, we are also going to conduct an intensive training/retraining and performance evaluation system for providers, as well as a rotation strategy between technicians and families. The monitoring file of the receiver is expected to collect information on intermediate potential causal steps that will facilitate attributing the observed alterations in outcomes to the intervention, i.e., demonstrating that the intervention was of sufficient magnitude and occurred in a temporal sequence consistent with the hypothesized impact. Additionally, the results from complex cluster-randomized controlled trials, due to the stringencies of the probability statements required, may not be generalizable and further effectivity studies may be needed. Recommendations for overcoming these difficulties include recourse to adequacy and plausibility arguments, combining different types of evidence, in turn limiting the occurrence of bias, confounding factors, and chance [90]. In this study, detailed impact, provision, utilization and coverage indicators will be collected, as described in Table 2.

7. Conclusions

Exclusive therapeutic strategies have important results in decreasing prevalence and intensity of infections, however, in a contaminated environment, reinfections rapidly occur. In one hand, WASH/Malaria community-based educational approaches may sustain the achievements of therapeutics by decreasing transmission and, on the other hand, a more accurate evaluation of the effect of community-based educational strategies in adequate IYCF may occur when the influence of infections is removed. The design of this study aims at investigating the effect of 1) exclusive therapeutics (in a focused and context-adapted test-and-treat approach), 2) therapeutics plus educational WASH/Malaria and 3) therapeutics plus educational nutrition strategies. This will allow to clarify key questions that could help improving the control strategies targeting infectious-related and nutrition-related anemia in the country.

Author Contributions: C.F. participated in the study design and structuring the manuscript. Â.S. participated in the study design and drafting the manuscript. H.B. participated in the study design and contributed to structuring the manuscript. J.L. contributed to the study design and drafting of the manuscript. M.B. participated in the study design and contributed to structuring the manuscript.

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Financial funders or material/facilities supporters play no role in the design, implementation and publication of the results of this study. Furthermore, considering that this study also incorporates a PHD thesis, taking place at the Institute of Public Health of the University of Porto, this project will also count on the technical and scientific support from the three mentors involved, namely Henrique Barros, Miguel Brito and João Lavinha, as well as the institutions they are affiliated to, respectively, the Institute of Public Health, the Faculty of Medicine, the University of Porto, the School of Health Technology of Lisbon and the Department of Human Genetics at the Ricardo Jorge National Institute of Health, in addition to CISA's researchers (the two first authors of this protocol). Thus, they were correspondingly involved in the design of this study protocol, and may also participate in data interpretation, the critical revision of the reports and manuscripts generated in addition to the afore mentioned CISA researchers.

The Health Research Center of Angola (CISA, translated) is the sponsor of this research project. This institution is located in Caxito, the capital of Bengo province (Angola) and its postal address is Estação Central Postal de Luanda, Apartado IV n°5547, Luanda. In addition to designing the study, this institution will be responsible for both implementation (data collection, analysis, and interpretation of data) and the publication of the generated results.

Ethical Declarations: After the explanation of the study, field technicians will provide an informative brochure to the caretakers. Thereafter, they will be asked to sign an informed consent, to formalize their acceptance and commitment to participating in this study. The collected data will be computerized and archived to ensure participant confidentiality and anonymity, and accessible only to the principal investigator and the research study coordinators. *In this study, the prevention of anemia through education is predicted to occur only as a result of interventions. They are expected to have an*

additive beneficial effect upon deworming. However, considering that the control arm will only be covered by the therapeutic component, this could constitute an ethical problem. Nevertheless, it should be considered that important improvements in Hb or prevalence of anemia's related etiologies have also been reported for exclusive therapeutic approaches. Additionally, all children testing positive for malaria, urogenital schistosomiasis and intestinal parasites, at any evaluation moment, will receive treatment according to the national therapeutic guidelines and specific bibliography. Furthermore, any children identified as suffering from severe illness will be sent to the emergency pediatric unit at Bengo General Hospital and children with sickle cell disease will be scheduled for a specific consultation with a CISA pediatrician in order to receive appropriate treatment and parental counselling. This study was approved by the Ethics committee of the Ministry of Health of the Republic of Angola. This study was registered at www.isrctn.com (number ISRCTN18101157) on 11/04/2016, registered retrospectively.

WHO Trial Registration Data Set: Primary registry and trial identifier number: ISRCTN: 18101157 (https://doi.org/10.1186/ISRCTN18101157); Date of primary registration: 11/04/2016; Source of monetary or material support: Calouste Gulbenkian Foundation, Banco de Fomento Angola, Special Program for Research and Training in Tropical Diseases and José Eduardo dos Santos Foundation; Primary sponsor: Health Research Center of Angola; Contact for public queries: claudia.videira@cisacaxito.org;

Public title: Anemia and its preventable etiologic agents in pre-school children from Bengo, Angola;

Scientific title: Efficacy of community educational interventions in nutrition and WASH/Malaria in reducing anemia in children under five, in the municipality of Dande—Angola;

Health conditions studied: Anemia and its etiologic agents;

Interventions: (1) Nutrition educational intervention plus screening and treatment of malaria, schistosomiasis and STH, (2) WASH and malaria educational intervention plus screening and treatment of malaria, schistosomiasis and STH and (3) exclusive screening and treatment of malaria, schistosomiasis and STH at the evaluation moments;

Key inclusion criteria: All children aged under 4, resident in hamlets with functional health posts;

Key exclusion criteria: Reported adverse reactions to albendazole and praziquantel and failing the baseline assessment and treatment;

Study type: Prevention;

Date of first enrolment: 15/06/2015;

Predicted date of the end of the study: 15/06/2019;

Target sample size: All children resident in hamlets with functional health posts will be considered eligible. Furthermore, there were 13 hamlets registered within the study area as having health posts, in which a density of 974 children was estimated using the CISA HDSS database; however, the functionality of those health posts needs further assessment in order to determine the clusters to be included and consequently the real sample size;

Recruitment status: No longer recruiting;

Primary outcome: Variation of hemoglobin levels between baseline, 6 and 12 months;

Key secondary outcomes: Variation of anthropometric indices, micronutrient deficiency and food diversity between baseline, 6 and 12 months.

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Abbreviations

ALB	Albendazole
CISA	Health Research Center of Angola (translated)
FPA	Food Photography Atlas
G6PD	Glucose Phosphate Dehydrogenase
HDSS	Health, Demographic and Surveillance System
HPLC	High-performance Liquid Chromatography
ID	Iron Deficiency
IDDS	Individual Dietary Diversity Score Indicator
IYCF	Infant and Young Child Feeding
PCR	Polymerase Chain Reaction
PZQ	Praziquantel
RCT	Randomized Controlled Trial
STH	Soil-Transmitted Helminth
UNICEF	United Nations Children's Fund
WASH	Water, Sanitation and hygiene
WHO	World Health Organization

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Paper V: Effectiveness of nutrition and WASH/malaria educational community-based interventions in reducing anemia and its etiologies in preschool children from Bengo, Angola: a cluster-randomized controlled trial.

Effectiveness of nutrition and WASH/malaria educational community-based interventions in reducing anemia in children from Angola: a cluster-randomized controlled trial

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Abstract

As far as we know, in Angola there is no published data regarding the effect of combining nutrition-specific and nutrition-sensitive approaches in the reduction of anemia in preschool children.

Thus, we implemented a cluster-randomized controlled trial to determine the effectiveness of two educational-plus-therapeutic interventions, namely Nutrition and WASH/Malaria, in reducing anemia, 1) comparing to a test-and-treat intervention and 2) comparing with each other. A block randomization was performed to allocate 6 isolated hamlets to 3 study arms. A difference-in-difference technique, using Fit Generalized estimating models, was used to determine differences between the children successfully followed in all groups, between 2015 and 2016.

We found no significant differences in anemia and hemoglobin variability between the educational and the control group. However, the WASH/Malaria group had 22.8% higher prevalence of anemia when compared with the nutrition group, having also higher prevalence of *P. falciparum*. Thus, our results

suggest that adding a 12-month educational Nutrition or a WASH/Malaria component to a test-and-treat approach may have a limited effect in controlling anemia. Possibly, the intensity and duration of the educational interventions were not sufficient to result in the amount of behavior change needed to stop transmission of infections and improve the general child feeding practices.

Background

In sub-Saharan Africa, controlling infectious morbidity through drug therapy alongside increasing micronutrient intake has been the most used approach to interrupt the processes by which infectious diseases cause anemia (such as blood loss, inflammation, hemolysis and blunted intestinal villi) and may increase the impact of health interventions [89, 90, 112]. However, controlling schistosomiasis, intestinal parasites and malaria exclusively through drug therapy may have limited sustainability in contaminated environment and/or in settings with inadequate water, sanitation and hygiene and malaria preventive practices and behaviors, because high re-infection rates may occur [106, 127, 175]. Thus, an educational component is essential to limit transmission.

A nutrition-sensitive approach could increase hemoglobin based on the assumption that providing health education and promoting adequate Water, Sanitation and Hygiene and malaria (WASH/Malaria) preventive practices would lead to improved knowledge and awareness of mothers/caretakers regarding the causes, prevention and treatment of urogenital schistosomiasis, intestinal parasite and malaria, in turn reducing inadequate practices and risk behaviors (child´s, mother´s and mother-to-child practices) and therefore reducing prevalence, incidence and re-infection rates of those diseases [106, 114, 127-129, 176].

Nevertheless, nutrition-specific approaches are crucial to address micronutrient deficiencies caused by inadequate Infant and Young Child Feeding Practices (IYCF), which could also benefit from increased sustainability if combined with a therapeutic approach. A nutrition educational intervention could reduce anemia based on the assumption that the intervention would improve the knowledge and awareness of mothers and caretakers regarding adequate feeding practices, which in turn could be translated into behavior changes resulting in improved nutritional diversity of the food consumed by the child, which could finally be reflected in decreased anemia and increased hemoglobin levels [73, 75, 76, 126, 177].

Previously we have designed a cluster-randomized controlled trial protocol aiming at determining the efficacy of educational community-based interventions in the reduction of anemia in preschool children, namely 1) nutrition and 2) WASH/malaria education, both combined with a test-and-treat approach [148]. However, at the pre-intervention assessment the number of eligible clusters were found to be significantly lower than expected, the baseline characteristics of the participants were

heterogeneous between the groups studied, and difficulties in assuring that interventions would be implemented under the ideal and highly controlled experimental conditions were anticipated. In the present study, we transitioned to an effectiveness cluster-randomized controlled trial. In doing so, small modifications were performed in the previous protocol, mainly in the main objective. Here, we primarily aim at testing whether a nutrition and WASH/Malaria educational, community-based interventions were able to reduce anemia in pre-school children from Bengo (Angola), under real-world conditions. A beneficial point resulting from this study design modification is the possibility of understanding if these interventions can produce effects under ecologically relevant conditions. On the other hand, lower internal validity and generalizability are expected to be associated to these alterations [178]. Three hypothesis are being investigated, namely: 1) if adding a WASH/Malaria educational component to a test-and-treat therapeutic approach result in higher reduction of anemia, when compared to an exclusive therapeutic strategy, 2) if adding a nutrition educational component to a test-and-treat approach result in higher reduction of anemia, when compared to an exclusive therapeutic strategy, and 3) if the addition of a WASH/Malaria educational component (to a test-and-treat approach) result in higher reduction of anemia, than the addition of a nutrition educational component.

Results

Participant flow and characteristics

At the end of the census approach, 1106 households were considered eligible and the ones with the presence of the caretaker at the moment of the census were invited to participate. Of those, 830 caretakers and 948 children from seven hamlets attended the evaluation day at their local health centers. Furthermore, before randomization, one cluster was excluded due to a very low number of eligible resident children (only 5 children); despite that they continued to be followed. The number of participants randomly assigned to each arm and evaluated is described in figure 1.

Despite randomization, we found differences in the baseline characteristics between arms, regarding the children completing the study. For instance, the control arm had higher proportion of girls and children with *S. haematobium* infections, while children in the nutrition arm had lower mean of hemoglobin levels and higher proportion of anemia, zinc deficiency, feeding frequency and green leaf consumption, and children in the WASH/Malaria arm had higher prevalence of Iron Deficiency and being infected with at least one intestinal/urogenital parasite (see Supplementary table S1). These variables were used to adjust the effectivity models for the heterogeneity in their distribution among study groups.

Dropouts occurred differentially between groups only regarding the mean of zinc levels (p-value=0.042).

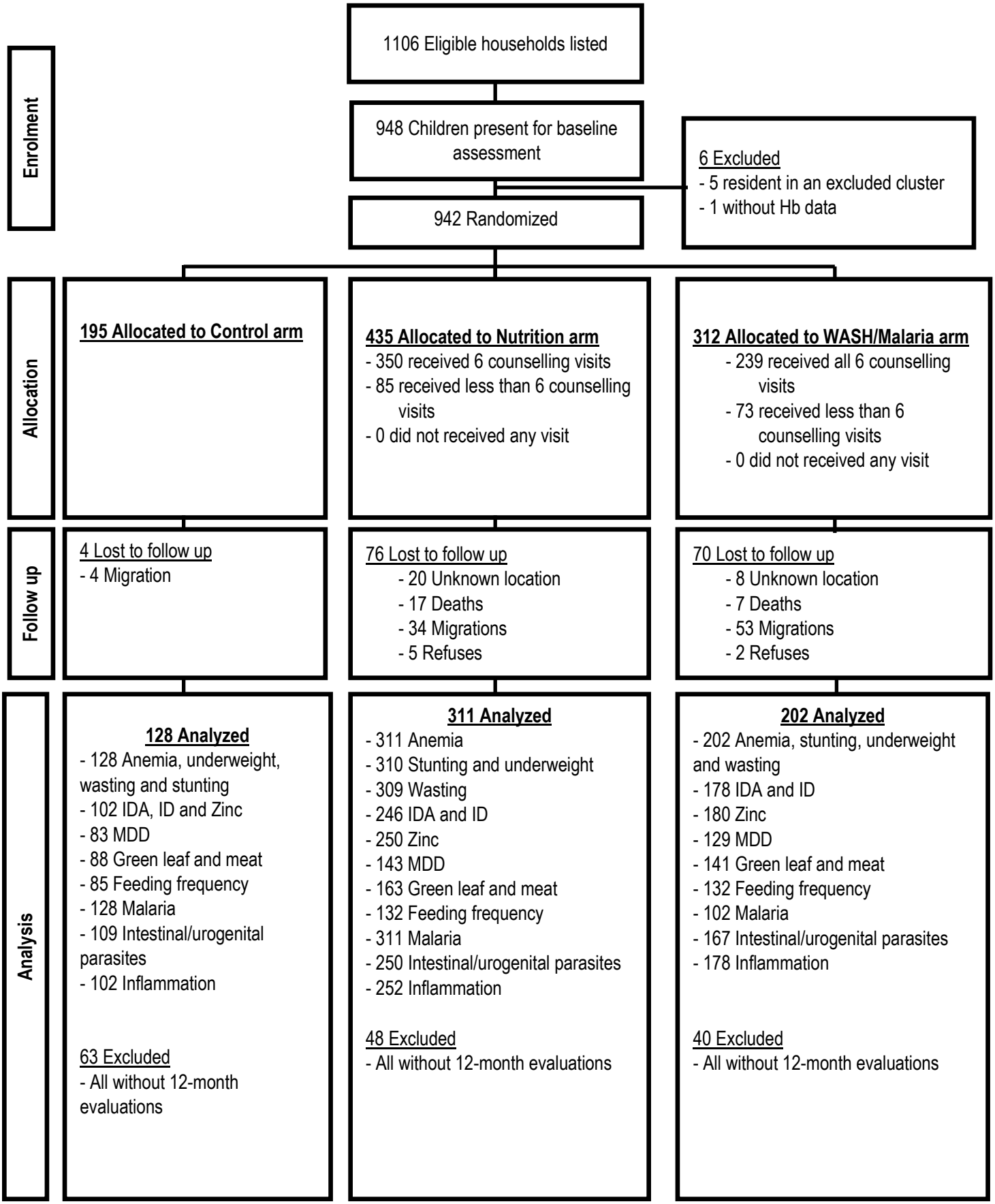

Figure 1 – Children follow up flowchart. Hb – Hemoglobin, IDA – Iron Deficiency Anemia, ID – Iron Deficiency, MDD – Minimum Dietary Diversity.

Outcomes and estimation

Our results from crude difference-in-difference models showed no statistically significant differences between the 3 interventions, regarding the reduction of anemia and increasing of Hb (see table 1). Nevertheless, differentiated variations between groups were found to occur in the prevalence of Iron Deficiency, Zinc deficiency and *P. falciparum* malaria (Wald chi-square p-value = 0.04, 0.04 and <0.001, respectively). Those differences occurring specially between the control and the nutrition arms (Wald chi-square p-value=0.077, p-value=0.015 and p-value=0.001, respectively).

When the regression models were adjusted, for the variables presenting differentiated prevalence profile at the baseline (and taking the control arm as the reference), we found that the nutrition group presented higher percentual enhancement in the prevalence of iron deficiency and lower enhancement of zinc deficiency and *P. falciparum* malaria prevalence (see table 1). On the other hand, the WASH/Malaria group presented lower enhancement of zinc deficiency prevalence.

Furthermore, when we compared the adjusted WASH/Malaria educational effect to the Nutrition educational effect, we observed that children in the WASH/Malaria group had 22.8% higher prevalence of anemia than the nutrition group. There were also higher prevalence of *P. falciparum* malaria and reported feeding frequency. We found no statistically significant associations between the variations in the outcomes and the number of domiciliary visits provided (see table 1).

Table 1 – Effectiveness of interventions in reducing anemia and their associated factors.

Type of models	**Crude models**	**Adjusted model 1**				**Adjusted model 2**		
Comparations	**Control vs. WASH/Malaria vs. Nutrition**	**Nutrition vs. Control**		**WASH/Malaria vs. Control**		**WASH/Malaria vs. Nutrition**		**WASH/Malaria vs. Nutrition Group with Interview score effect**
Statistic evaluators	Wald chi-square p-valu	% enhance.	DDI (p-value)	% enhance.	DDI (p-value)	% enhance.	DDI (p-value)	DDI (p-value)
Health primary outcome								
Hb (Mean)	0.29	2.0	+0.151 (0.468)	0.7	0.023 (0.912)	-1.3	-0.160 (0.371)	0.023 (0.403)
Anemia (% yes)	0.22	-17.2	-0.424 (0.273)	0.3	0.263 (0.498)	22.8	**+0.641 (0.050)**	-0.048 (0.332)
Health secondary outcomes								
IDA (% yes)	0.11	50.5	+0.468 (0.351)	24.2	+0.276 (0.606)	-27.3	-0.228 (0.594)	0.017 (0.776)
Iron deficiency (% yes)	**0.04**	71.6	**+0.866 (0.032)**	-5.3	+0.278 (0.498)	-47.5	-0.664 (0.144)	0.088 (0.258)
Zinc deficiency (% yes)	**0.04**	-94.9	**-3.336 (0.006)**	-103.7	**-2.126 (0.088)**	169.5	+1.194 (0.117)	0.081 (0.411)
Weight (Mean)	0.88	0.6	+0.258 (0.291)	0.6	+0.281 (0.253)	0.0	-0.029 (0.886)	-0.006 (0.843)
Stunting (% yes)	0.29	23.3	+0.196 (0.610)	40.4	+0.217 (0.575)	11.8	0.043 (0.89)	-0.041 (0.425)
Underweight (% yes)	0.55	-	-	-	-	-	-	-
Wasting (% yes)	0.44	-	-	-	-	-	-	-
P. falciparum (% yes)	**<0.001**	-317.7	**-3.052 (0.001)**	-669.0	-0.637 (0.433)	569.1	**2.495 (0.013)**	-107 (0.351)
At least one intestinal/urogenital parasite (% yes)	0.79	-129.1	-0.073 (0.867)	-6.9	-0.316 (0.445)	49.8	0.244 (0.510)	-0.061 (0.301)
Inflammation (% yes)	0.35	-	-	-	-	-	-	-
Behavior Secondary outcome								
Green leaf intake (% yes)	0.66	2,3	+0.261 (0.516)	15,9	+0.368 (0.385)	11.7	+0.103 (0.763)	0.009 (0.874)
Meat cons. (% yes)	0.72	-12,0	-0.230 (0.587)	-10,6	-0.262 (0.516)	-1.1	-0.058 (0.877)	0.048 (0.358)
Feeding frequency (Mean)		-15,4	-0.345 (0.164)	-15,8	-0.073 (0.769)	2.6	**+0.416 (0.049)**	0.006 (0.864)
MDD (% yes)	0.73	-	-	-	-	-	-	-

Hb – Hemoglobin, IDA – Iron deficiency anemia. *difference-in-difference Wald chi-square, # Moderate-to-severe. **Models 1** - Yit = β0 + β1 Time + β2 Group + δ (Time × Group) + β3 Gender + β4 Age + β5 Zinc Deficiency + β6 Feeding frequency + β7 Green leaf intake + β8 At least one intestinal/urogenital parasite +ε. **Model 2** (nutrition-WASH/Malaria) and 2 (nutrition-WASH/Malaria with interview scores) - Yit = β0 + β1 Time + β2 Group + δ (Time × Group) + β3 interview score + β4 Gender + β5 Age + β6 Zinc Deficiency + β7 Feeding frequency + β8 Green leaf intake + β9 At least one intestinal/urogenital parasite +ε

To further understand those results, link them to the interventions and assist with the interpretation, we performed additional ancillary analysis in each group (from the baseline to 12-month follow up) (described in table 2).

Ancillary analysis

Comparing pre-and-post intervention moments (baseline to 12-month follow up variations)

In this study, an increased hemoglobin level (0.05 g/dl, 0.27 g/dl and 0.13 g/dl, respectively for the control, nutrition and WASH/Malaria arms) and a decreased prevalence of anemia (5.5%, 14.2% and 5.4% lower) were observed in all study arms, between the baseline and the 12-month follow up (Table 2). However, significant increased hemoglobin level and decreased anemia prevalence were only observed in the nutrition arm.

Iron Deficiency Anemia (IDA) prevalence was found to be significantly reduced either among the children in the control (from 24.5% to 10.8%) and WASH/Malaria arms (from 21.9% to 13.5%), while no significant reduction on iron deficiency prevalence was observed (among the children with hemoglobin level ≤ 11g/dl). The reduction of zinc deficiency prevalence was significant in the nutrition arm, decreasing from 12.8% to 3.6%, from the beginning to the end of the study (see table 2). Furthermore, despite that decreased wasting was observed in the nutrition arm, the prevalence of stunting was found to increase 14.2% among the children in this group. Stunting among the children in the WASH/Malaria group also increased (16.8%).

Regarding infections, a significant variation in the prevalence of *P. falciparum* malaria was observed in all arms (from 6.3% to 21.1% in the control arm, from 5.8% to 2.3% in the nutrition arm and from 1.5% to 6.4% in the WASH/Malaria arm). Furthermore, the prevalence of children infected with at least one intestinal/urogenital parasite was also found to increase in all arms (32.1% in the control, 19.2% in the nutrition and 30.5% in the WASH/Malaria arm) (see table 2).

Table 2 - Variation in the primary and secondary outcomes among children completing the study, between the baseline and 12-month follow up

	Control				**Nutrition**				**WASH/Malaria**			
Outcomes	T0	T2	Dif.	P-value	T0	T2	Dif.	P-value	T0	T2	Dif.	P-value
Primary outcomes												
Hb (Mean (SD))	11.2 (1.1)	11.3 (1.3)	0.05	0.706*	11.0 (1.4)	11.3 (1.3)	0.27	**0.002***	11.3 (1.4)	11.4 (1.1)	0.13	0.210*
Anemia (% yes)	39.1 (50/128)	33.6 (43/128)	-5.50	0.392**	50.5 (157/311)	36.3 (113/311)	-14.2	**<0.001****	38.6 (78/202)	33.2 (67/202)	-5.4	0.215**
Secondary outcomes – Nutritional state												
IDA (% yes)	24.5 (25/102)	10.8 (11/102)	-13.73	**0.016****	22.4 (55/246)	19.9 (49/246)	-2.4	0.539**	21.9 (39/178)	13.5 (24/178)	-8.4	**0.032****
Iron deficiency (% yes)	12.7 (13/102)	7.8 (8/102)	-4.90	0.359**	14.2 (35/246)	19.5 (48/246)	5.3	0.105**	24.2 (43/178)	18 (32/178)	-6.2	0.185**
Zinc deficiency (% yes)	2 (2/102)	4.9 (5/102)	2.94	0.453**	12.8 (32/250)	3.6 (9/250)	-9.2	**<0.001****	4.4 (8/180)	2.8 (5/180)	-1.7	0.581**
Weight gain (Mean (SD))	9.2 (2.4)	12.1 (2.3)	2.84	**<0.001***	9.0 (2.5)	11.9 (2.1)	2.9	**<0.001***	9.1 (2.3)	12.0 (2.1)	2.9	**<0.001***
Stunting (% yes) #	29.7 (38/128)	37.5 (48/128)	7.81	0.143**	27.4 (85/310)	41.6 (129/310)	14.2	**<0.001****	22.3 (45/202)	39.1 (79/202)	16.8	**<0.001****
Underweight (% yes) #	20.3 (26/128)	18 (23/128)	-2.30	0.678**	18.1 (56/310)	15.8 (49/310)	-2.3	0.382**	21.3 (43/202)	14.9 (30/202)	-6.4	0.066**
Wasting (% yes) #	9.4 (12/128)	6.3 (8/128)	-3.10	0.454**	7.1 (22/309)	2.3 (7/309)	-4.8	**0.003****	10.9 (22/202)	5.4 (11/202)	-5.5	0.052**
Secondary outcomes – infections												
P. falciparum (% yes)	6.3 (8/128)	21.1 (27/128)	14.85	**<0.001****	5.8 (18/311)	2.3 (7/311)	-3.5	**0.043****	1.5 (3/202)	6.4 (13/202)	4.9	**0.021****
At least one intestinal/urogenital parasite (% yes)	16.5 (18/109)	48.6 (53/109)	32.11	**<0.001****	10 (25/250)	29.2 (73/250)	19.2	**<0.001****	22.8 (38/167)	53.3 (89/167)	30.5	**<0.001****
Inflammation (% yes)	51 (52/102)	41.2 (42/102)	-9.80	0.174**	39.3 (99/252)	40.5 (102/252)	1.2	0.850**	48.9 (87/178)	44.9 (80/178)	-3.9	0.494**
Secondary outcomes – Feeding practices												
Green leaf intake (% yes)	12.5 (11/88)	29.5 (26/88)	17.05	**0.014****	31.9 (52/163)	49.7 (81/163)	17.8	**0.002****	17.7 (25/141)	37.6 (53/141)	19.9	**<0.001****
Meat consumption. (% yes)	39.8 (35/88)	27.3 (24/88)	-12.50	0.117**	33.7 (55/163)	17.2 (28/163)	-16.6	**<0.001****	42.6 (60/141)	25.5 (36/141)	-17.0	**0.003****
Feeding frequency (>=3)	57.6 (49/85)	75.3 (64/85)	17.65	**0.014****	70.5 (93/132)	77.3 (102/132)	6.8	0.243**	59.1 (78/132)	67.4 (89/132)	8.3	0.177**
MDD (% yes)	13.3 (11/83)	16.9 (14/83)	3.61	0.664**	9.1 (13/143)	7.7 (11/143)	-1.4	0.824**	17.1 (22/129)	17.1 (22/129)	0.0	1.000**

SD – Standard deviation, Hb – Hemoglobin, MDD – Minimum Dietary Diversity * Students' t, ** McNemar Test.

Comparing the 1st and 6th visits for behaviour change

More than 70% of the participants within the educational interventions successfully received 5-to-6 visits (70.8% of the households in the WASH/Malaria group and 71.8% in the Nutrition group). Furthermore, statistically significant increase in the proportion of households with latrine, clean latrines, clean environment and observed clean nails of the caretaker were observed in the WASH/Malaria group and significant increased weekly consumption of cereals, food from animal sources, legumes, vegetables, fruits and in the minimum feeding frequency was also observed in the Nutrition group (supplementary table S2).

Discussion

Results from our crude and adjusted difference-in-difference models suggest that implementing two semesterly rounds of an exclusive test-and-treat therapeutic approach have no statistical significative difference in reducing anemia and increasing Hemoglobin, that when combining this approach with 6 monthly domiciliary counselling visits addressing adequate child nutrition or WASH/Malaria practices. Considering the positive results reported by others, and the fact that our process indicators suggest the occurrence of some degree of behavior change in both educational intervention groups (supplementary table S2), these effectiveness results could suggest that, 1) the therapeutic component of the intervention played in fact the major role and educating had a residual effect, or 2) not all educational actions have been translated into changed behavior and in consequence the needed sequence of beneficial results happened incompletely (see supplementary Figure F1 and F2) and/or 3) the educational actions lacked the required intensity (number of monthly visits and duration of each session) and duration of the educational actions (only 12-months) to translate into significant changes at the primary outcomes [102, 158].

In fact, the way educational interventions are expected to impact in the primary outcomes constitute a long and complex pathway of actions, that may be subject to effect modifications among different populations. For instance, the therapeutic component (implemented in all study arms) was expected to clear infections that could be causing anemia; further allowing educational interventions to either improve WASH/Malaria practices that could reduce the infection transmission and allow the natural recovery of Hemoglobin (in the WASH/Malaria arm); or to improve feeding practices that could lead to higher quantity and quality of diet and improve the Hemoglobin status of children in the absence of infections (in the Nutrition arm). Nevertheless, at the end, all tree interventions were expected to increase hemoglobin levels and reduce anemia prevalence, besides improving other nutritional outcomes between the pre- and post-evaluation moments. Thus. plausibility arguments are recommended to be used as evidence of impact when evaluating the effect of real-world complex interventions [179]. Accordingly, we have analysed our data by protocol, rather than by intention to

treat, to allow combining different types of evidences able to assist the interpretation of our results and to provide a more comprehensive discussion following the assumption mentioned above [159].

For instance, instead of observing reduced prevalence of infections over a 12-month period (between the baseline and the 12-month follow), we observed a significant increase in the prevalence of having at least one intestinal/urogenital parasite (mainly *G. lamblia*, *S. haematobium* and *A. lumbricoides*) in all arms, and in the prevalence of *P. falciparum* malaria among the children within the control and WASH/Malaria arms, contrary to other studies [106, 107, 111, 175, 180, 181]. Assuming that baseline infections were cleared by the test-and-treat approach, this may associate with the occurrence of frequent/repetitive contact of children with highly contaminated environments, in turn influenced by the children age (as the wider mobility of older children may increasingly expose them to sources of contamination), which the WASH educational component wasn't able to prevent [14, 182]. That is corroborated by the similar longitudinal infectious profile of both Control and WASH/Malaria arms, and by the absence of significative differences in those variations in regression models. In the literature, while some studies report that adding chemotherapy to health education and sanitation can reduce the prevalence, intensity and/or incidence of *A. lumbricoides*, *T. trichiura*, hookworms or schistosomiasis and improve the knowledge, attitude and preventive practices toward those infections in, others report no significant effect [106-108, 111]. Similarly, malaria, educational packages were mentioned to improve the knowledge and preventive practices and treatment-seeking behavior, while integrating community case management and the possession nets were reported to lower the prevalence and incidence of fever in under-five years old children [109, 110, 114, 115].

Also, adjusted models showed that the Nutrition group had significantly less *P. falciparum* prevalence than the Control group. This, could be related to seasonal malaria and ecological conditions [101, 102]. Interestingly, the Nutrition group had also less zinc deficiency and more iron deficiency (consistent in crude models). To note that zinc is a trace element associated to the nutrition-dependent immune response (also called as nutritional immunity), being confined into cellular compartments during inflammatory events to decrease its concentrations and bioavailability to the pathogen [40-42]. Also, zinc levels can affect both tissue and/or systemic level responses to iron deficiency (affecting local iron regulatory proteins and/or the normal function of an iron regulatory hormone called hepcidin) [33, 38, 40, 53, 160, 163-166]. Thus, we speculate that the reduction of *P. falciparum* malaria and zinc deficiency prevalence may have influenced each other, in turn affecting the prevalence of iron deficiency. Nevertheless, our results don't allow to determine to which extent the educational approaches could have contributed differently to this fact.

Regarding the effect of educating for adequate IYCF practices, despite that a significant increase in the prevalence of reported consumption of green leaf in the previous 24h (18.2%) was documented between the baseline and the 12-month follow up, children at the Nutrition group were reported to have consumed significantly less meat (-16.9%) and lower feeding frequency (-0.26) at the end of the study, when compared to the baseline. Additionally, the intervention failed at increasing the prevalence of Minimum Dietary Diversity (MDD) observed at the baseline. Those facts suggest that the educational component of the intervention may have had positive impact in some behavioral secondary outcomes but not in all of them (assumptions supported by our process indicators described in supplementary Table S2). Others reported that educational programs in nutrition may lead to children being fed more green leafy vegetables, nutrient-dense thick foods at lunch and meeting the dietary requirements for energy, iron and zinc more frequently and improved ability of mothers to identify malnutrition, despite that methodological variations may lead to different results [69, 70, 75, 76].

Two main operational limitations must be considered when interpreting our results. First, relates to the limitations documented during the implementation of the Test-and-Treat approach (performed in all study groups). Here, limitations in the implementation of the active screening and treatment of infections in the community was associated to failure to obtain samples (or to obtain the needed volume), samples collected in inappropriate containers (which may have introduced contaminations and misestimated frequencies), impossibility to perform Kato Katz in diarrheal samples (limiting the diagnosis of helminths), diagnosis made from single samples, failure of caretakers/children's to attend treatment visits or consultations, non-supervision of all therapeutic dosage intake, difficulty in relocating households, and inadequate filling of treatment records by technicians (making the success of some treatments being inconclusive). Secondly, providing bed nets, soap and bleach to all groups may have encouraged caretakers to use it, potentially confounding the results of the control and nutrition arms. There was variation in the infectious profile of each cluster and that should also be taken into consideration.

It is reported that when moving from efficacy to effectiveness studies, we may also move to lower degree of generalizability [178, 179]. Here, considering that randomization did not produced absolutely comparable groups, as differences regarding some baseline characteristics were observed between groups (i.e. the prevalence of some outcomes was not diluted within the clusters), generalizability is limited [183]. Thus, we recommend that the interpretation of results should be restricted to our settings and study populations. Furthermore, to use the difference-in-difference analysis in groups differing significantly at the baseline may wrongly suggest that changes occurred in one group and not in the other [184]. To minimize the impact of that issue in our results we have adjusted the difference-in-difference models, accounting for variables whose baseline prevalence or value was statistically

different between the groups being compared, as performed by others in similar studies (see supplementary Table S1) [153]. When interpreting our results, the reader must also consider that we have used a fixed number of clusters (of fixed size), and that it is reported that when the results of cluster randomized trials (with less than 40 clusters), are analysed using generalised equations there is an increased risk of inflated type I error (i.e, false positive findings) [184]. Nevertheless, some design aspects, commonly associated with efficacy research studies, were sustained in the effectiveness study implemented here, such as standardized design, structured interventions, focus on specific primary outcomes (while the improvement in behavior was kept as a secondary outcomes) and randomized assignment to interventions were still being implemented. Thus, our study may represent a hybrid between efficacy and effectivity, potentially allowing for a more systematized (real-life intervention) results, comparatively to traditional effectiveness studies.

Methods

All methods in this study were carried in accordance to relevant guidelines and regulations, following both national regulatory norms and standards, as also international recommendations.

Trial design

This study is an open (unblinded), parallel, cluster-randomized controlled trial, with 3 arms.

Participants

Eligibility criteria

Children younger than 3 years old, resident in the selected hamlets, who were evaluated at the pre-intervention assessment, were considered eligible.

Recruitment, follow up and counselling visits

The initial assessment of participants occurred between March and May of 2015 (baseline that included 948 children), while the follow-ups occurred between November and December 2015 (6-month follow up that included 524 children) and between July and August of 2016 (12-month follow up that included 660 children). The counselling moments have intercalated those evaluation moments in 2 rounds of 3 counselling visits. The first occurred between June and October 2015 (included 610, 576 and 574 of the caretakers) and the second round occurred between January and June of 2016 (included 561, 574 and 563 of the caretakers).

Settings and locations where the data were collected

The data was collected in 7 administratively and geographically isolated hamlets with health facilities, located within the CISA Health and Demographic Surveillance System (HDSS) study area, and that were found to provide daily primary care at the pre-intervention assessment. The DHSS area comprehend essentially the communes of Úcua, Caxito and Mabubas, in turn located within the Dande municipality of the Bengo province (in Angola). Detailed characterization of demography, socioeconomic aspects, causes of death and epidemiology of some diseases have already been published [20, 22, 118-124].

Interventions

Therapeutic (test-and-treat) component

This component was included in all study arms and was conducted and implemented at the evaluation moments. Briefly, *P. falciparum* infections were screened and treated at the evaluation sites, while urogenital schistosomiasis and/or intestinal parasites were diagnosed at the CISA´s laboratory and treated at specific domiciliary visits (in the case of treatments with albendazole) and/or hospital-based consultations (in the case of treatments with praziquantel) [148]. A standard treatment protocol, following the national therapeutic guidelines and specific bibliography, was used. Furthermore, children diagnosed with sickle cell disease were referred to the Anemia Patient Follow-up Consultation, held at the Bengo General Hospital.

Educational component

As previously published, caretakers of children allocated to the educational interventions in Nutrition or WASH/Malaria have received six domiciliary, personalized counselling sessions [148]. Those educational visits were conducted from June of 2015 and June of 2016), having 3 evaluation moments being implemented before they start (at the baseline), after 3 of those visits (first follow up) and after the sixth visit (second follow up). Briefly, counselling to the educational intervention groups targeted eight main topics. Regarding the nutrition group, the main topics were breastfeeding, complementary feeding, weekly adequate food diversity, appropriate number of meals, adequate amount of food, responsible feeding, food and disease, and food safety and hygiene. On the other hand, the WASH/Malaria group was counselled on insecticide-treated nets (ITNs) usage, reducing mosquito breeding sites, prevention of open sky defecation, latrine cleaning, adequate hand washing, healthy backyard environment, adequate water availability, treatment, transportation and storage and adequate personal hygiene as previously described [148].

Socio-economic and demographic data and biological specimen collection and laboratory testing

The sample collection and processing have been previously described in detail [148]. Briefly, a standardized questionnaire was administered by trained interviewers to mothers or caretakers, aiming at collecting socio-economic and demographic information, as well as information regarding pregnancy and breastfeeding, complementary feeding practices, health care, food security, WASH and malaria practices [132, 133]. Weight, height and mid-arm circumference were measured and used to calculate anthropometric indices for the diagnosis of undernutrition, according to WHO standards [185]. Blood was collected by venous puncture according to WHO recommendations for neonates and young children and used for 1) the diagnosis of *P. falciparum,* through Rapid Diagnostic Tests (*SD BIOLINE* Malaria Ag *P.f*/*P.v*®, Standard Diagnostics, Inc., Republic of Korea) and 2) biochemical analysis for the quantification of blood levels of hemoglobin (using an Hemocue ® Hb 301 System, Angelholm, Sweden) and serum levels of ferritin, Protein-C- Reactive and Zinc (using an automated autoanalizer (BT1500) from Biotecnica Instruments S.p.A, Rome, Italy) and CRP turbidimetric latex ®, Ferritin® and Zinc® kits from Quimica Clínica Aplicada S.A., Tarragona, Spain) [136]. The diagnosis of intestinal parasites in stool samples was performed using Kato-Katz technique and Parasitrap® kits (Biosepar, Germany) and urogenital schistosomiasis was diagnosed by urine filtration, using Whatman® Nuclepore™ membranes (diam. 25mm, pore size 12µm, polycarbonate, Merck, Germany) [138-140].

Outcomes

Primary outcomes comprised only health indicators, namely increase in hemoglobin levels and reduction of anemia prevalence, as described by Fançony *et al* 2019 [148]. Secondary outcomes comprised health and behavior indicators for the estimation of impact evaluations, while process indicators (which comprised either reported or observed practices or behaviors collected at the counselling visits) were used to conduct process evaluations. The complete list of outcomes and their analysis strategy within each arm is presented table 3.

Table 3 – Outcomes and analysis performed in this study.

Variables	**WASH/Malaria**	**Nutrition**	**Control**
3) Primary outcome: *1.2) Impact on health measured at the evaluation moments**			
Hemoglobin levels (g/dl)	x	x	x
Anemia prevalence (%)	x	x	x
4) Secondary outcomes: *2.5) Impact on health measured at the evaluation moments**			
Iron deficiency anemia (%)	x	x	x
Weight (kg)	x	x	x
Stunting (%)	x	x	x
Underweight (%)	x	x	x

Having at least one intestinal/urogenital parasite (%)	x	x	x
P. falciparum (%)	x	x	x
*2.6) Impact on behaviour reported at evaluation moments**			
Minimum dietary diversity (MDD) (%)	x	x	x
Green leaf consumption (in previous 24h) (%)	x	x	x
Meat consumption (in previous 24h) (%)	x	x	x
Children sleeping under bednets in the previous night (%)	x	x	x
*2.7) Impact on behaviour observed by the technician at the WASH/Malaria educational sessions***			
Nº of domiciliary counselling visits delivered (%)	x	NA	NA
Having bednet in the children's bed (%)	x	NA	NA
Latrine ownership (%)	x	NA	NA
Having water to wash hands in the latrine (%)	x	NA	NA
Clean latrine (%)	x	NA	NA
Backyard environment with garbage (%)	x	NA	NA
Backyard environment with loose animals (%)	x	NA	NA
Backyard environment with still water (%)	x	NA	NA
Caretaker having clean nails (%)	x	NA	NA
2.8) *Impact on behaviour: reported by caretakers at the nutrition educational sessions***			
Nº of domiciliary counselling visits delivered (%)	NA	x	NA
Weekly cereal consumption (%)	NA	x	NA
Weekly seeds consumption (%)	NA	x	NA
Weekly Milk and derivatives consumption (%)	NA	x	NA
Weekly Food from animal sources consumption (%)	NA	x	NA
Weekly Eggs consumption (%)	NA	x	NA
Weekly Legumes consumption (%)	NA	x	NA
Weekly Vegetables consumption (%)	NA	x	NA
Weekly Fruits consumption (%)	NA	x	NA
Feeding frequency (%)	NA	x	NA

x Evaluated; *Mean and frequency variation between baseline and the 12-month follow up; ** frequency variation between the first and the sixth counselling visits; NA – Non applicable.

Sampling strategy

The sampling strategy chosen for this study was a non-probabilistic (convenience) sampling [148]. Eligible children were listed and invited to participate using a census approach, within a fixed number of clusters. This strategy was adopted because variations in the density of eligible children were expected and the real density in each cluster was uncertain.

Randomization

For this study we used a block randomization, where hamlets (considered as cluster units) were randomly allocated to each study arm. At the end of the pre-evaluation assessment, only 6 hamlets were found to be eligible and were randomized to the study arms. For the randomization, the names of the hamlets were written down on pieces of paper, placed in a bag and successively removed. The

first two papers removed were attributed to the Nutrition arm, the following two to the WASH/Malaria arm and the next pair to the control arm. This process was carried out by the study researchers.

Statistical analysis

SPSS software (version 25.0, International Business Machines Corporation, Pittsburgh, PA) was used for statistical analyses.

Prevalence was calculated as the frequencies of the outcome over the total number of samples with valid results and prevalence reduction (PR) was calculated as the difference between the proportion of prevalence at the baseline *vs* 12-month after the beginning of the study.

To investigate the differences in the baseline characteristics, between the children completing the study and the ones dropping out after 12-months, we used Chi-square Test for the categoric variables and T Student for the continuous variables. Logistic regression models were used to analyze interactions between the study arms and variables (using dropouts as dependent variables) and determine if losses were differentiated between arms. Thus, the existence of differences in the baseline characteristics between study arms (for children completing the study) was investigated using ANOVA and Chi-square, respectively to continuous and categoric variables.

Students´ t and McNemar tests were used to determine longitudinal variations on the primary and secondary outcomes between baseline and the end of the study (respectively at the pre and post-interventional moments) within each arm.

To determine the differences in the crude variations between the three study arms, the difference-in-difference technique was performed using Fit Generalized estimating models. In summary, linear regression models were used to estimate the mean hemoglobin level and weight variations and logistic regression models were used to estimate the variation on the prevalence of anemia and other secondary binary outcomes. In each model, independent variables were time (0=T0 and 1=T1), the group, and group*time interaction. Outcomes presenting significant longitudinal variations within each arm, or significant crude differences when comparing study arms, were selected to be further inspected for significant differences in adjusted models. Adjustment was conducted by including secondary outcomes whose prevalence was found to be differentially distributed among the three arms at the baseline (considered as potentially confounding variables) and also age (found to influence the occurrence of anemia in previous studies). The interaction term (represented by DDI) indicates whether there were statistically significant differences in the adjusted variation of T0 to T1 between groups, as previously described by Mahfuz *et al* 2016 [153]. The control group was used as

the reference group when determining the effectiveness of the therapeutic-plus-educational intervention, comparatively to an exclusive therapeutic (test-and-treat) intervention, while the nutrition group was used as the reference group when determining the effectiveness of the WASH/Malaria educational intervention (further accounting for the number of interviews conducted in both educational interventions). The quantification of the crude percent of enhancement in the hemoglobin level and in the prevalence of anemia and associated factors was calculated as described by Mahfuz *et al* 2016 [153].

Process indicators were collected at every counselling visits (from a total of six visits) conducted in nutrition and WASH/Malaria arms. Changes in the proportion of adequate behaviour or practices were calculated between the first and the sixth visits, using McNemar tests. Adequate household behavior/practices in the WASH/Malaria arm included observed bednet in the children's bedroom, clean latrine, latrine with water to wash hands (either with current or still water mechanisms), clean backyard environment, namely without garbage, loose animals, still waters and caretaker with clean nails. In the nutrition group, adequate behavior/practices included the reported weekly consumption of cereals, seeds, milk and milk derivatives, food of animal origin, eggs, legumes, vegetables, other fruits and the minimum feeding frequency. "Sustained behavior/practice" was considered to be a behavior or practice observed in both first and sixth visits and "Changed behavior" was considered to be an altered behavior or practice between the first and sixth visits.

Ethical considerations

After the explanation of the study, an informative brochure was given to the mothers or caretakers. Thereafter, they were asked to sign an informed consent in order to formalize their acceptance and commitment to their children participation. The collected data were computerized and archived to ensure the confidentiality and privacy. Children with positive malaria, urogenital schistosomiasis and intestinal parasites diagnostic tests were treated with Artemether-Lumefantrine, albendazole (ALB) and Praziquantel (PZQ) at the baseline, and 6 and 12 months follow ups (according to a therapeutic protocol approved by a pediatrician and the director of Bengo General Hospital). Follow up consultations were scheduled for children with severe undernutrition and anemia at the undernutrition/malnutrition department at the Bengo General Hospital. Mothers not taking their children to those consultations were visited to understand the reasons for that and to explain to them the importance of their compliance. This study was approved by the Angolan Ministry of Health Ethics Committee.

Harms

No harms have been documented during the implementation of this study.

Author Contributions

CF - Conceptualized the research question and participated in the design, implementation and supervision of the field work. Also, performed the statistical analysis, drafted the initial manuscript and revised the subsequent versions.

AS – Participated in the design, implementation and supervision of the field work and critically reviewed the manuscript.

JL - Provided scientific and technical support, and critically reviewed the manuscript.

HB - Helped conceptualizing the research question, oriented the statistical analysis and the manuscript structuration.

MB - helped conceptualizing the research question, participated in the overall study design and critically reviewed the manuscript.

All authors approved the last version of the manuscript to be submitted and are responsible for this work

Competing Interests Statement

The authors declare no competing interests.

Registration

This study is registered with the title: Efficacy of community educational interventions in nutrition and WASH/Malaria in reducing anemia in under 5 children, in the municipality of Dande – Angola, at www.isrctn.com (https://doi.org/10.1186/ISRCTN18101157), with the trial identifier number ISRCTN: 18101157. The date of primary registration is 11/04/2016.

Protocol

The original protocol can be assessed at https://www.ncbi.nlm.nih.gov/pubmed/30764549.

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Conclusions

In this study, adding a 12-month educational nutrition or a WASH/Malaria component to a test-and-treat approach had no greater beneficial effect reducing the prevalence of anemia and increasing the level of hemoglobin than an exclusive therapeutic approach. Possibly, the intensity (number of counselling visits and length of each session) and duration of the educational actions may haven’t

resulted in the amount of behavior change needed to stop transmission or improve the general child feeding practices, as reported by others.

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