# Efficacy of biofortified (yellow) cassava on Serum Retinol in nigerian pre-school Children

Ibukun Afolami

**Propositions**

1. Yellow cassava increases serum retinol when regularly consumed by children.
   *(this thesis)*

2. Malaria parasitaemia in human plasma interferes with the determination of vitamin A status independently of inflammation.
   *(this thesis)*

3. Gene editing technologies hold great promise for the treatment of cardiovascular diseases.

4. The incentives and benefits attached to academic peer review need to be reviewed.

5. The truth about any reality being observed is inherent in the reality itself and does not depend on the observer's perception.

6. Doing science in Nigeria is an experience worthy of documenting in a novel.

Propositions belonging to the thesis, entitled

Efficacy of biofortified (yellow) cassava on serum retinol in pre-school Nigerian children

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Wageningen, 31 August 2021

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# Efficacy of biofortified (yellow) cassava on serum retinol in pre-school Nigerian children

Ibukun Afolami

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*"All the world is a laboratory to the inquiring mind."*

Dr. Martin H. Fischer (1879-1962)

To Steve
& Folake

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# Chapter One

General Introduction

**The problem of vitamin A deficiency and the justification for biofortification**

Vitamin A deficiency (VAD) is a micronutrient deficiency caused by the insufficient intake of vitamin A [1] . Vitamin A intervention programs such as food fortification and vitamin A supplementation became a global agenda around the 1990s [2–4]. Since then, several countries have adopted these interventions to reduce deficiency in their populations. During this period, a significant reduction in VAD prevalence was shown in all regions except south-east Asia and sub-Saharan Africa, where VAD prevalence has barely decreased [5].

One important factor contributing to the unchanging high prevalence of VAD in sub-Saharan Africa is the nature of African diets, which are heavily composed of cereal and root staples [6] . Due to their low incomes, many poor households are unable to afford foods such as fruits, pulses, and animal products, which ordinarily contain significant amounts of the required vitamins and micronutrients [7]. Furthermore, important micronutrients available in vegetables consumed by this population are lost, partly due to processing [8–11], cooking methods [12,13] and bio-unavailability [14–16].

In 2002, the idea of biofortification was conceived as a complementary intervention to address VAD [17,18] as well as other micronutrient deficiencies, especially in low and middle income countries in sub-Saharan Africa and Asia. Using this approach, locally produced staples were enriched with specific micronutrients using conventional breeding methods. Since then, various teams of nutritionists have worked with breeders to develop nutritional breeding targets for micronutrients of public health significance such as vitamin A, zinc, and iron [17]. Subsequently, commonly consumed staple crops in the regions, such as cassava, maize, sweet potato, rice, wheat, beans, and pearl millet were bred to produce micronutrient-dense varieties of these staples through the process of biofortification. These staples were later tested for nutritional efficacy in the forms in which they are usually consumed. The objective of the efficacy trials was to determine if the nutrients bred in the staples can be absorbed and utilized at sufficient levels in the target populations, such that they can improve micronutrient status [17].

**Vitamin A absorption and transportation, bioavailability, and bioconversion.**

For any provitamin A intervention food to be efficacious, provitamins from such a food would need to be available for absorption into the bloodstream i.e. bioavailable. Provitamins A are metabolically inactive forms of Vitamin A, which naturally exists in plant-based foods and are converted into active

vitamin A forms in a process known as bioconversion. Bioconversion is therefore an important aspect of provitamin A metabolism.

Ingested retinoids and carotenoids are absorbed mostly in the small intestine. Retinoids and carotenoids in foods are released from the food matrices in the lumen of the small intestine, where they undergo emulsification with dietary fatty acids and bile acids. The emulsification process converts the insoluble retinoids (i.e. retinol, and retinaldehyde) and carotenoids into more aqueous forms, making them more easily absorbed by enterocytes [19]. In the enterocytes, retinol is converted into retinyl-ester [20], while β-carotene is cleaved into two molecules of retinal by the enzyme β-carotene 15,15′monoxygenase enzyme (BCMO1) [21]. The retinal is then converted to retinol by retinol reductase enzyme **(figure 1)**. In humans, a significant fraction of the carotenoids and almost all the retinol absorbed into the enterocytes is transported into the lymphatic system as part of chylomicrons, where they are transported to the liver and extrahepatic tissues [22]. In the liver, most of the absorbed dietary vitamin A is transferred to hepatic parenchymal cells or hepatocytes when chylomicron remnants are metabolized by the liver [23]. Here, (in the plasma membrane of hepatocytes or early endosomes), the retinyl esters are hydrolysed into retinol and retinoic acid [19]. The retinol is subsequently transferred to the endoplasmic reticulum, where retinol binding protein (RBP) is found in high concentrations. The binding of retinol to RBP apparently initiates a translocation of retinol-RBP complex from the endoplasmic reticulum to the Golgi complex, followed by secretion of retinol-RBP complex from the cells [24]. Retinol is then transferred from hepatocytes to stellate cells [25] which contain between 90 – 95% of liver retinol. About 98% of stellate cell vitamin A is in the form of retinyl esters, packed together in cytoplasmic lipid droplets [26]. In the blood, retinol-RBP complex is transported to extrahepatic tissues through the mediation of the hormone, transthyretin [27].

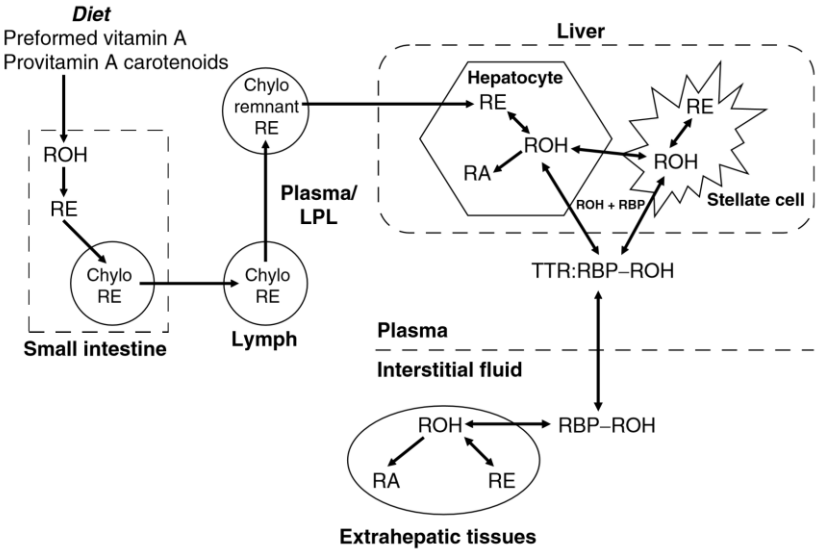


**Figure 1**: Whole body vitamin A metabolism (adapted from Cifelli *et al.,* 2007)

Measuring bioavailability in provitamin A -rich foods is a daunting task, as scientists are faced with methodological challenges [28]. First and foremost, serum retinol, although presently the most widely-used indicator of vitamin A status [33], is not very sensitive in responding to intervention effects [29] mainly because it is homeostatically controlled by liver enzymes which work to keep the concentration constant in the blood [28]. As a result, some previous provitamin A efficacy trials did not detect any change in serum retinol concentrations in some populations, even after consumption of provitamin A-rich foods [30–32]. Furthermore, populations with high infection prevalence often record a corresponding high prevalence of inflammation, leading to a transient reduction in serum RBP – the protein responsible for transporting retinol in the blood [33,34]. However, this often leads to an underestimation of serum retinol concentrations and an overestimation of vitamin A deficiency. These two factors have been commonly reported as important confounders of serum retinol concentrations, which may mask the true effect of provitamin A on vitamin A status during efficacy trials in some populations [35].

Isotope dilution techniques and dose response tests are examples of more recent methodologies that are now being used to estimate bioavailability and bioconversion during efficacy trials [28]. These methods have proven to be more sensitive in detecting intervention effects because they exploit other principles of provitamin A metabolism apart from serum retinol

concentrations [36]. For example, isotope dilution techniques measure total body retinol pool (including liver stores) and dose response tests measure apo-RBP during depletion [28]. These qualities make these methodologies more appropriate than using serum retinol concentrations in determining bioefficacy of provitamin A during interventions. However, both methods have limitations during infection or inflammation [28]. In the presence of inflammation, absorption of RBP is reduced and excretion increased. Hence, when using the isotope dilution methods, the ratio of isotope enrichments of retinol in serum to liver is affected [28].

Some studies have shown that bioconversion factors vary widely depending on the food matrices [37–41]. Evidence from these studies thus question the use of fixed factors in the estimation of retinol equivalents from provitamin A intake. Specific conversion factors have been calculated for fruits [42], biofortified staples [32,37,38] and green leafy vegetables [39,42]. Specifically, with regards to provitamin A biofortified foods, recent studies have shown that provitamin A from cassava is readily bioavailable with bioconversion factors as low as 3.7 to 1 [43].

**The conceptual framework for a successful biofortification programme**

The goal of biofortification is to increase micronutrient status in deficient populations. From the outset, it was generally understood that for biofortification programmes to achieve this overall goal, implementation programmes would need to accomplish smaller goals which were specific, measurable, achievable, and time-bound (SMART). These goals have been captured in three implementation phases described by Saltzman *et al*. [17] as the discovery, development, and delivery phases of implementation **(figure 2)**. Presently, the biofortification agenda is approaching the delivery phase. So far, about 12 different biofortified staples are available in over 30 countries [18], providing additional micronutrients. In sub-Saharan Africa, provitamin A sweet potato, maize and cassava have been developed and are now available.

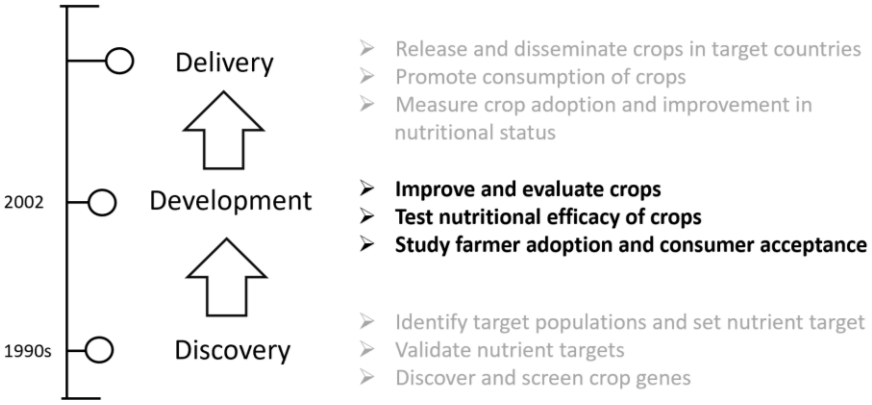


**Figure 2:** Conceptual roadmap for biofortification programme: Adapted from Saltzman *et al,* 2013

**Cassava as a choice staple for biofortification in Africa**

Cassava roots are important calorie sources for about 65% of the African population, contributing between 300kcal to 600kcal of energy intake per person daily [44]. This justifies the adoption of cassava as one of the choice staples for provitamin A biofortification in Africa, where cassava production is used almost exclusively for consumption as food in different forms such as fresh roots, dried roots, pasty product and granulated products [44]. Nigeria is the largest producer and consumer of cassava in Africa and the world, producing over 59 million metric tonnes per year [45]. The quantity of cassava produced in Nigeria alone comprises about 36% of total production in Africa [45]. Based on these facts, Nigeria was selected as the most appropriate country to conduct an efficacy trial on provitamin A biofortified cassava.

**The evidence in support of biofortification**

There is ample evidence available so far, supporting the nutritional efficacy of provitamin A biofortified foods. In South Africa, β-carotene-rich orange-fleshed sweet potato improved the vitamin A status of primary school children[46]. In Zambia, provitamin A biofortified maize consumption increased vitamin A liver reserves in rural Zambian children [37]. In 2012, an efficacy trial was conducted on biofortified cassava in school-aged Kenyan children 5 – 13 years old, who were fed cooked cassava porridge prepared with biofortified cassava. Results showed that the consumption of the provitamin A cassava porridge modestly improved serum retinol [47]. However, the study had two limitations: on one hand, mortality and morbidity attributable to vitamin A deficiency is more of a problem in pre-school or under-five children rather than in school-aged children. Secondly,

the cassava meals used in the study were not the usual recipe or dish commonly consumed in the West African population. It was thus important to ensure that future efficacy trial on provitamin A biofortified staple was conducted among pre-school children, who could potentially benefit most from vitamin A interventions. It was thus important to ensure that future studies were designed in such a way that the intervention foods prepared with biofortified cassava, were the local dishes commonly consumed by the target population to facilitate future implementation and to increase adherence.

**Efficacy trial with biofortified (yellow) cassava in Nigeria: A justification for the present study**

To accommodate for the translation of findings from the Kenyan efficacy study to the Nigerian context, a second efficacy trial with biofortified cassava was designed. This study constitutes the core of this PhD thesis. Preparations for this study began in Nigeria in 2015. Prior to this, the cassava varieties – TMS 593 and TMS 419, used to prepare the foods consumed in the experimental and control groups of the study, were propagated at the International Institute for Tropical Agriculture (IITA) in Nigeria. Prior analysis showed that the yellow cassava variety contained about 9µg/g β-carotene at maturity. The study was conducted in Telemu, located in Olaoluwa Local Government Area (LGA) of Osun State **(figure 3)**. Participants were recruited from three adjacent communities - Ilemowu and Asamu within the same Local Government Area (LGA) **(figure 3)**, and were registered in a preschool specifically established for the study, where they were taught but also fed breakfast and lunch.

One notable improvement of this study over the Kenyan study, was the use of the yellow cassava to prepare local dishes, commonly consumed in the community. This approach solved the problem of diet acceptability, which is expected when a new food product is introduced to a population [48]. However, it also introduced a confounder because there were other non-biofortified sources of carotenoids in the dishes consumed. It was therefore important to measure and account for these sources of carotenoids in all the foods consumed. Furthermore, Nigeria is notably a palm oil-consuming country, and the study population was no exception to this. Palm oil is a very rich source of carotenoids, although between 80% to 99% of the beta-carotene in palm oi is often destroyed during prolonged heating, which is a usual cooking practice in south-west Nigeria [49]. There was thus a need to control for palm oil intake. This was achieved by feeding the children with vegetable oil with zero carotenoid at the preschool and additionally by measuring palm oil intake consumed outside the preschool.

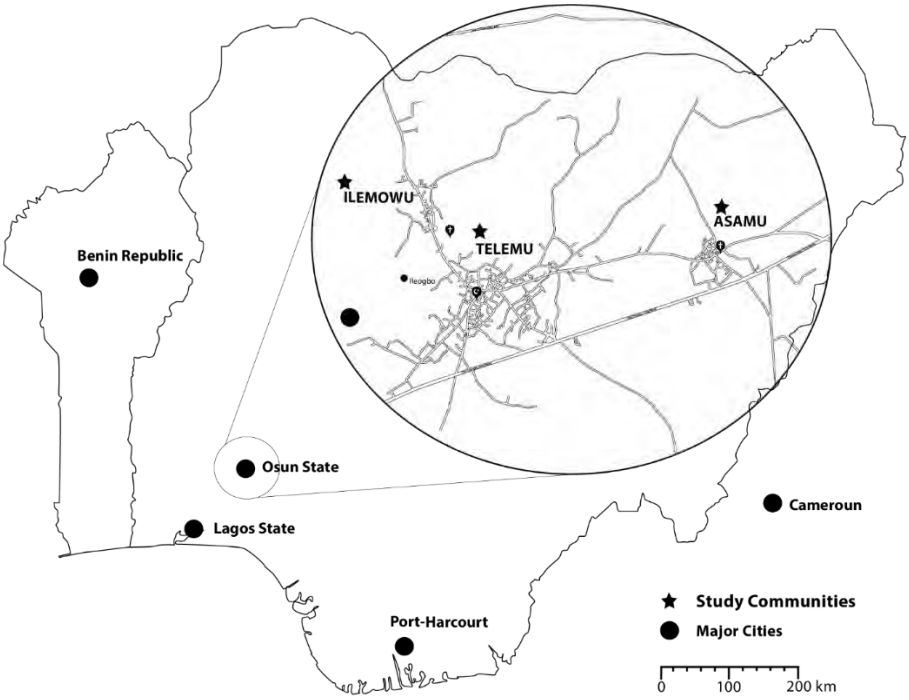


**Figure 3:** Map of study location

**Provitamin A biofortification and Gut integrity**

Previous studies have shown that vitamin A can improve the epithelial integrity of the gut (reference). A direct or indirect effect of provitamin A biofortification in principle, may achieve the same result. We hypothesized that provitamin A from biofortified foods may show some functional benefits of improving gut epithelial integrity or reducing bacterial translocation and the risk of infection.

**Study Objectives**

The objectives of the studies in this thesis are presented in the table below, based on the individual chapters presented in the thesis.

**Table 1**. Study objectives

| Thesis Chapter | Study design, population & outcome | Objectives |
|---|---|---|
| Chapter 2 | Randomized Controlled Trial.<br>Population: Preschool children, 3-5 years, attending a study-specific preschool.<br>Study outcomes: serum retinol, serum β-carotene, haemoglobin, lactulose mannitol ratio. | i. To determine the efficacy of yellow cassava consumption on serum retinol concentration.<br>ii. To determine the effect of yellow cassava consumption on serum β-carotene concentrations.<br>iii. To determine the effect of yellow cassava consumption on haemoglobin concentration.<br>iv. To determine the effect of yellow cassava consumption on gut permeability. |
| Chapter 3 | Cross-sectional study.<br>Population: preschool children, 3-5 years, attending a preschool designed for the study.<br>Study outcome: intestinal permeability ratio (LMR, LRR, SRR) | i. To determine the effect of voided urinary volume on intestinal permeability ratios in apparently healthy children. |
| Chapter 4 | Randomized Controlled Trial and cross-sectional study.<br>Population: preschool children, 3-5 years, attending a preschool designed for the study. | i. To determine median RAE intake between YC and WC groups during and after intervention. |
| | Cross-sectional study. | ii. To determine the potential contribution of yellow cassava to total dietary retinol activity equivalent (RAE) intake if white cassava is replaced by yellow cassava among pre-school Nigerian children. |
| Chapter 5 | Cross-sectional study.<br>Population: preschool children, 3-5 years in a malaria-endemic population.<br>Study outcome: retinol binding protein | i. To compare three vitamin A adjustment methods in Nigerian pre-school children at risk of malaria infection.<br>ii. To determine the effect of malaria and inflammation on retinol binding protein concentration among preschool children in a malaria-endemic population. |

**Outline of the thesis**

This thesis is primarily an efficacy trial of biofortified cassava. In this study, the contribution of biofortified cassava to serum retinol was tested in a randomized controlled trial in Nigerian preschool children living in communities endemic to malaria and other pathogenic infections. The report of the efficacy trial is presented in chapter two, with an overall aim of testing the effect of daily consumption of biofortified cassava on serum retinol. As a secondary objective, gut permeability of participants was assessed, based on the rationale that an overtly permeable gut may "leak" retinol.

While studying the gut permeability of the children, it was observed that the estimated value for gut permeability varied with the volume of urine voided by participants. This observation and its implication on the assessment of gut permeability is first discussed in chapter three.

In chapter four, the dietary vitamin A intake of the children is reported. The objective here, was to measure usual vitamin A intake during and after the intervention and to estimate what would be the percentage contribution of biofortified cassava to vitamin A intake if biofortified cassava is eventually adopted to replace the white cassava in the children's diet. It was expected that an intervention of this kind, which fed children with pro-vitamin A-rich foods twice a day, would change the feeding pattern of participants. Dietary intake was therefore measured at a second time point, after the intervention when the children had returned to their normal feeding pattern.

Chapter five focuses on the effect of malaria and other infections on retinol binding protein concentrations. This chapter also compared different adjustment methods currently being used to correct for the confounding effect of infection on serum retinol. Based on the understanding that serum retinol concentrations may be confounded by malaria and other pathogenic infections, it was of interest to study the effect of these on serum retinol concentrations.

Finally, in chapter six, the main findings and their public health implications are discussed.

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# Chapter Two

## Daily consumption of pro-vitamin A biofortified (yellow) cassava improves serum retinol concentrations in pre-school children in Nigeria: a randomised controlled trial

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**ABSTRACT**

**Background:** Vitamin A deficiency is a public health problem in sub-Saharan Africa. Provitamin A biofortified (yellow) cassava has the potential to contribute significantly to improve vitamin A status, especially in populations that are hard to reach with other strategies.

**Objective:** The study aimed at determining the efficacy of biofortified cassava to improve vitamin A status of Nigerian preschool children.

**Design:** An open label randomized controlled trial was conducted in South-western Nigeria. In total, 176 pre-school children (3-5 years old) were randomized into two parallel arms comprising an experimental group (n=88), fed foods prepared from biofortified (yellow) cassava, and a control group (n=88), fed foods prepared from white cassava, twice a day, six days a week for 93 days.

**Result:** A total of 159 children completed the trial (yellow cassava group, n=79; white cassava group, n=80). Children consumed 220.8 µg/day and 74.1 µg/day retinol activity equivalents from intervention foods in the yellow and white cassava groups, respectively. The treatment effect on serum retinol concentrations at the end of the feeding trial was 0.06 µmol/L (95% CI: 0.004, 0.124), after adjustment for baseline retinol concentrations, inflammation, and asymptomatic malaria status. No significant treatment effects were detected for serum β-carotene (adjusted effect: 3.9%; 95% CI: -0.6%, 8.6%) and gut permeability (adjusted effect: 0.002 (95% CI: -0.089, 0.092), but a significant effect was detected for haemoglobin concentrations (adjusted effect: 3.08 g/L (95% CI: 0.38, 5.78).

**Conclusion:** Daily consumption of β-carotene from biofortified cassava improved serum retinol and haemoglobin concentrations modestly in Nigerian preschool children.

**Keywords:** Vitamin A; Biofortification; Cassava; Pre-school children; Nigeria.

## INTRODUCTION

Vitamin A deficiency (VAD) is a global public health problem with high prevalence especially in developing regions of the world. Biofortification is a food-based approach aimed at reducing the global burden of micronutrient deficiencies, including VAD. Currently, orange flesh sweet potato, yellow cassava, and orange maize are being used as biofortified staples to deliver pro-vitamin A across large populations in Uganda, Nigeria and Zambia, amongst others [1]. Efficacy trials conducted with some of these crops have shown modest to ample effects so far, proving the principle that provitamin A biofortified foods can contribute significantly to total body pools of retinol [2–5].

Nigeria, with an estimated population of 200 million, is ranked as the largest producer and consumer of cassava in the world, producing approximately 55 million metric tons annually [6]. Despite multiple intervention programs to reduce VAD, ~30% (10 million) of under-five children in the country are still categorized as vitamin A-deficient based on the latest, yet outdated national survey [7]. Cassava can therefore be considered as a suitable food choice for the delivery of provitamin A through biofortification in Nigeria [8].

Estimating the effect of a vitamin A intervention is challenging because serum retinol concentrations do not sensitively reflect changes in status when in the sufficient range [9]. Moreover, vitamin A status can be highly confounded by inflammation [10] and malaria [11] The presence of malaria has been reported to lower serum retinol concentration independently of inflammation, though the mechanism of this remains unclear [12,13]. Furthermore, as a result of an increase in vitamin A supplementation coverage and fortification efforts, it has become more difficult to prove the efficacy of a food-based approach such as biofortification, especially in preschool children that are targeted by multiple vitamin A programs. Therefore, the Retinol Isotope Dilution (RID) technique to estimate body retinol pools, which captures vitamin A status over the full status range, has emerged as an alternative method to evaluate treatment effects [14,15]. Several efficacy trials with provitamin A rich foods have shown the usefulness of the RID technique in that respect [2,3,16,17].

In this study, we determined the efficacy of provitamin A biofortified (yellow) cassava in pre-school children living in a malaria-endemic region in Nigeria. Our pre-planned primary outcome was the total body pool of retinol using the RID technique. However, despite our efforts to implement this technique with methodological rigor and informed advice from international experts, we were unable to detect the isotope in collected blood samples. Alternatively, we therefore used the difference in change in serum retinol concentration between treatment and control group as the

modified primary outcome of the study. The study was registered at www.clinicaltrials.gov (NCT02627222).

## METHODS

### Study population and subjects

The study was an open label randomized controlled trial (RCT). Study participants were recruited from three adjacent rural communities (*Telemu, Ashamu and Ilemowu*), in Osun State, South-West Nigeria. These communities were purposively selected, based on pre-established criteria, such as rural setting, safety, likelihood of marginal vitamin A status, community goodwill, ease of logistical operations, and proximity to the cassava harvest site. The study area belonged to the rainforest agroecological zone with two rainy seasons from March to July and September to October. The annual rainfall in the country is approximately 1,379 millimetres, with an annual minimum and maximum temperature of 21.1 and 31.9 °C, respectively [18]. The last national survey in 2004 showed a prevalence of VAD of 22.4% in the study area, which was the highest in the region [7]. VAD prevalence is highest in the northern part of Nigeria,; however, we could not conduct the study in this location because of the safety of staff and study resources. In the South-West, there is a high *Plasmodium sp*. entomological inoculation rate of 110 infective bites/person/year [19,20]. Osun State has approximately 4,009,800 people, predominantly from the Yoruba tribe, who are mostly subsistent farmers growing cassava, yam and maize [21].

The study was conducted between December 2015 to May 2016. Ethical approval in Nigeria was obtained from the University of Ibadan/ University College Hospital Ethics Committee (UI/EC/14/0426). State Government approval was obtained from the Ministry of Health in Osun State (OSHREC/PRS/569T/53), as well as positive advice from the Medical Research Ethics Committee of Wageningen University, the Netherlands (14/37). Study participation was on voluntary basis and confidentiality of all the obtained information of the participants was maintained throughout the study. Written informed consent, and assent on behalf of the child, was obtained twice from each participant's parent(s) or guardian(s): before the screening, and before study commencement.

All eligible children in the communities (n=568) were invited to participate **(figure 4)**. Children were screened for eligibility using the following inclusion criteria: (1) willingness to participate; (2) no visible sign of sickness; (3) older than 3 years and less than 5 years before study commencement; and (4) plasma haemoglobin (Hb) > 70g/L. Serum retinol binding protein (RBP) was also measured during the screening phase of the study to enable stratified treatment allocation. Most of the children were

not enrolled in any school at the beginning of the study. We set-up a pre-school, specifically for the study. Two hundred and sixty-two screened participants then entered a run-in phase and received a daily ration of control (white) cassava during this period. Children who were frequently absent or were unable to consume ≥80% of the age-specific target meal portion were excluded. During the run-in phase, all children received prophylactic treatment for malaria (Lumefantrine/Artemether), common intestinal helminth infection (Albendazole, 300mg) and schistosomiasis (Praziquantel, 300mg). Medications were administered approximately two weeks before baseline measurements to eliminate potential factors that could bias vitamin A status estimates at baseline. A total of 176 pre-school children, 3-5 years old were finally enrolled in the study.

The Nigeria national vitamin A supplementation program has a wide coverage. There was the possibility that recruited children might have just received vitamin A supplements from the national program, which would have introduced possible confounding in our study. We therefore monitored the July 2015 supplementation programme in the participating communities and kept a record of all supplemented children within the study location. A period of four months elapsed between the date of the last vitamin A supplementation and commencement of the intervention. The intervention also ended before the next round of supplementation. As a result, we hypothesized that the intervention would prevent a decline in body retinol pools to a certain extent over time, rather than increase body retinol pools.

**Study design and procedures**

The study comprised two parallel groups: the treatment and control groups. In the treatment group (n=88), children were fed foods prepared with yellow cassava; in the control group (n=88), children were fed the same menus but the foods were prepared with white cassava. Both the white and the biofortified (yellow) cassava were grown under the supervision of HarvestPlus/International Institute for Tropical Agriculture (IITA) in Ibadan, Nigeria. The varieties of cassava used in the study were TMS 419 (white cassava) and TMS 07/0593 (yellow cassava). The TMS 07/0593 variety was released in Nigeria in 2013 and showed to contain a concentration of ~9 μg/g β-carotene at maturity. Fresh cassava roots were harvested and transported to the research site twice per week. Trained cooks prepared the cassava daily according to standardized recipes (see online Supplementary methods) and under supervision with either yellow or white cassava; *moinmoin* and *garri* for breakfast; and cassava *eba* with *okra* or *ewedu* vegetable soup and stew (with meat) for lunch. Both groups were fed twice a day, breakfast and lunch, 6 days per week, for a total of 16 weeks **(figure 5)**.

The pre-planned primary outcome of the study was the difference in total body retinol pool between the yellow and white-cassava groups to be established by RID technique. On day 7, (**figure 4**), all participants were administered 0.4mg of stable $^{13}C_4$ retinyl acetate tracer, which had been formulated into capsules before the commencement of the study. This was followed by a 14-day equilibration period to enable the labelled tracer to mix with the existing unlabelled body retinol pool (tracee) as applied in other paediatric populations [2,22]. Baseline blood was then collected on day 21, following an overnight fast. At this point, children who successfully passed the run-in phase, and met the eligibility criteria, were randomized using a stratified-block design. Since initial body retinol pools are strong determinants of the response in the study outcome, participants were blocked based on their serum RBP concentration, determined during the screening phase of the study. For this, children were ranked according to their serum RBP concentrations and divided into tertiles of low, medium, and high RBP values. Subsequently, blocks of 4 and 6 were used to randomize children to the two intervention groups within each RBP stratum. Randomization was performed by an independent member of the team, who was not involved in the fieldwork. Since it was practically impossible to blind participants and research assistants to either the standard treatment (yellow cassava) or placebo treatment (white cassava), the study was carried out as an open-label trial.

After baseline measurements and randomization, feeding commenced according to intervention allocation. Feeding locations for the two groups were physically separated from each other, to prevent cross-over between groups. During feeding sessions, the foods were weighed by research assistants into serving containers with known weights, then served immediately to participants. Feeding was constantly monitored by field assistants, and weights of all leftovers were recorded. On day 140 (**figure 4**), 0.4mg of stable $^{13}C_{10}$ retinyl acetate tracer was administered, followed by another 14-day tracer/tracee equilibration. Endline blood samples were collected between days 154 and 157

We pre-estimated an initial pool size of 12,600 µg retinol; a high dose vitamin A supplementation of 60,000µg, 4 months before baseline;; vitamin A absorption/retention of the labelled dose of 0.76; and a fractional catabolic rate of 1.85%/day. Assuming a conservative retinol:β-carotene conversion factor of 1:7 [23], we estimated 150 µg RAE/day as the minimum amount to be consumed by every child in the intervention. Based on these assumptions, we estimated a difference in total body retinol pool size of 4224 µg between groups following intervention, with a standard deviation of 8,594µg, $\alpha$=0.05 and 80% power. Assuming a drop-out rate of 15%, we estimated a required sample size of n=81 in each study group.

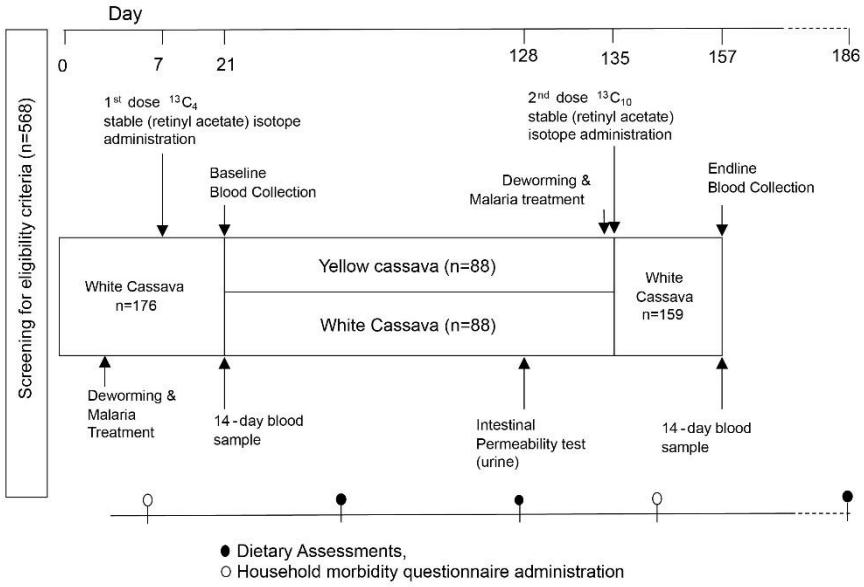


**Figure 4:** Study design

**Preparation of stable isotope**

A stock solution of $^{13}C_4$ or $^{13}C_{10}$ retinyl acetate (Cambridge Isotope Laboratories Inc, Tewksbury, MA, USA), verified for purity and isotopic composition by an external laboratory, was prepared by dissolving 120 mg of each compound in 25 ml ethanol in a volumetric flask. Subsequently, α-tocopherol (~2µg/mg retinyl acetate) was added as an antioxidant to the stock solution, and the ethanol was evaporated in the dark under nitrogen gas. Hozol oil (46.68 g) was then added to the residue to make a $^{13}C$ retinyl acetate concentration of 2.36 mg/ml. Subsequently, 0.17 mL doses were pipetted into opaque gelatin capsules (CapsuGel, Bornem, Belgium). Capsules were prepared at the Division of Human Nutrition and Health, Wageningen University, the Netherlands, and stored at 4°C until transportation to the study site and subsequent administration to participants under strict supervision. The amount of retinyl acetate solution in the capsules was verified for each capsule based on capsule weight. Eight capsules were analysed in duplicate by High Performance Liquid Chromatography (HPLC) and shown to contain 0.37 ± 8 mg of retinyl acetate. Another set of ten capsules was analysed after having been transported to the study location, stored at the field during the intervention, and return transported to Europe. These capsules were analysed by Liquid Chromatography-Mass Spectrometer (LC-MS/MS) in the Lietz' laboratory at the University of Newcastle upon Tyne, UK and contained 0.39 mg of $^{13}C_{10}$-retinyl acetate.

**24-Hour Recall Data Collection**
In the last week of the intervention, the mean intake of vitamin A and energy was estimated in 162 children from foods consumed within and outside the pre-school facility, using the multiple pass quantitative 24-Hr dietary recall method [24]. Repeated recalls were conducted on non-consecutive days for 30% of the children to account for intra-individual variation.

**Blood collection, processing and biochemical assessments**
Whole blood samples were collected by venepuncture into heparinized vacutainers (Becton and Dickinson, California, USA) by phlebotomists from the University College Hospital, Ibadan, Nigeria. Vacutainers were stored immediately in dark containers on ice and were transferred to the field laboratory located next to the preschool for further cooling, centrifugation, aliquoting and field-specific analysis under yellow light illumination conditions. All samples were processed and stored in liquid nitrogen at -196°C within 12 hours of collection until shipment. Samples were shipped frozen on dry ice to Wageningen University, The Netherlands where they were stored at -80°C until analysis.

Concentrations of CRP and Hb (20µL whole blood aliquot) were measured in the field at screening, baseline and endline by immuno-turbidimetric method, using a QuickRead-Go CRP/Hb) instrument (Orion Diagnostica, Espoo, Finland). Field analysis of CRP and Hb were conducted to immediately identify and treat critical cases of sick children. The instrument was calibrated with Hb and CRP controls obtained from the manufacturer. Plasma concentrations of RBP, ferritin, soluble transferrin receptor, C-reactive Protein (CRP), and α-1 acid glycoprotein (AGP) were simultaneously determined using a combined simple sandwich Enzyme-Linked Immunosorbent Assay (ELISA) technique in the VitMin laboratory, Wilstaett, Germany[25].

**Small intestine permeability test**
Towards the end of the study (**figure 4**), small intestine permeability was assessed in participants (n=155) using a multiple sugar gut permeability test [26]. For this, 40g each of lactulose and sucrose; and 20g each of mannitol and rhamnose was weighed into a clean measuring beaker, 1 litre of potable water was added and the sugars were stirred to dissolve. After voiding, each child was administered a 25ml portion containing 1.0g each of lactulose and sucrose and 0.5g each of rhamnose and mannitol. Subsequently, 100-200mls of potable water was administered to induce urination. Urine was collected during 90 minutes after sugar dosage and the time interval between sugar dosage and urine collection was noted. Urine weight and specific gravity were measured immediately after collection, using a weighing scale (Kern & Sohn, D-72336, Germany) and

urinalysis strip (Surescreen diagnostics, Derby, UK) respectively. Sample aliquots (5ml) were stored at -20°C between the time of collection and analysis. Urinary lactulose, mannitol, sucrose and rhamnose were measured by gas chromatography, using the method described by Jansen *et al*. (1986) [27].



**Determination of serum retinol concentrations**

Serum retinol concentration was measured by HPLC (Thermo Scientific Accella LC system; Thermo Fisher Scientific, Massachusetts, USA), whereas $^{13}C$ retinol isotope ratios were determined by LC-MS/MS at the Division of Human Nutrition and Health, Wageningen University, the Netherlands based on the method described by Oxley *et al* [28]. Both baseline and endline serum samples were analysed simultaneously to reduce inter-assay variation. Samples were thawed, homogenized and pipetted into Kimax tubes. Subsequently, 0.5ml serum, 0.9% physiologic salt (NaCl; 0.9w/v% in water), and 1000µl ethanol (with added retinyl acetate as an internal standard) were added to the samples and extracted twice with 2ml hexane. The hexane layers were pooled and aerated to dryness under nitrogen, using a freeze drier (Osterode, Germany). The extracted residue was dissolved in 125µl of methanol-1-butanol (for HPLC analysis) or ethyl-acetate (for LC-MS analysis), vortexed and transferred into HPLC vials for analysis. All sample preparation was conducted under subdued yellow light. Retinol and β-carotene were separated on a C18 reversed-phase column (Vydac 201TP52, Grace, Columbia, Maryland, USA), using a gradient elution technique. Analytes were detected at 325nm (retinol and retinyl acetate) and 450nm (carotenoids) on a photodiode array with multiple wavelength detector. HPLC quantitation was performed using the EZChrom Elite version 3.2.2 SP2 software (Agilent Technologies). Unfortunately, we were not able to detect clear peaks in isotope enriched retinol in serum samples analysed by LC-MS/MS, either at baseline or at endline. This was confirmed in the Lietz' laboratory at the University of Newcastle upon Tyne, UK. Therefore, we could not estimate the total body retinol pools of the children.

**Determination of carotenoids in food**

Samples of each recipe, as consumed, were collected randomly every week, during the intervention period. Each meal was collected in opaque containers and homogenized using a kitchen blender. Five millilitres of Butylhydroquinone (BHT) was added as a preservative to the samples during homogenization. All food samples were stored in opaque plastic jars at -20°C at the field site. After the intervention, samples were shipped to Wageningen University, and stored at -80°C until analysis. At the point of analysis, the different food samples were pooled together into composite samples representing three stages of the intervention to detect

any existing variation in the carotenoid content of the meals over the intervention period. Carotenoids in the foods were analysed using the same HPLC system described for serum samples. To extract the carotenoids from the foods, we mixed 2g homogenized food sample, 0.2g magnesium carbonate, 5mL deionized water, and 1000mL ethanol (with added retinyl acetate as internal standard), and extracted 3 times with 20mL methanoltetrahydrofuran (1:1 vol:vol%), using a rod mixer (Polytron PT 20 OD; Kriens/Luzern) until the residue was colorless. Extracts were filtered on a glass funnel with filter paper (Whatman grade 1, GE healthcare life Sciences); the combined filtrates were transferred to a 50-mL volumetric flask and made up to volume with methanoltetrahydrofuran (1:1 vol:vol%). Then, 4 mL filtrate with 1mL 10% NaCl solution was transferred to a 10-ml glass stoppered centrifuge tube (Kimax, Kimble Chase), and carotenoids were extracted 3 times with 1.5ml petroleum-ether containing 0.01% butylated hydroxytoluene. The combined ether fractions were evaporated under nitrogen at 358°C. The residue was dissolved in 2 mL methanol/butanol (60/40 vol:vol%), and 1mL was injected into the HPLC system. Carotenoids were separated on a Vydac 201TP52 column by gradient elution and monitored at 450 nm on a photodiode array detector. Runtime was 20 min per sample. We measured the summed content of trans and cis β-carotene, the predominant provitamin A carotenoid in yellow cassava

**Malaria assessment**

Malaria infection was measured by rapid dipstick tests based on the detection of histidine-rich protein-2 and *Plasmodium*-specific lactate dehydrogenase (CareStart, product code G0121, Access Bio Inc., Monmouth Jct., NJ, USA). In addition, *P. falciparum*-specific DNA was extracted from erythrocytes and malaria parasite counts were detected at the Amsterdam Medical Centre, the Netherlands, based on published protocol [29].

**Anthropometry**

Height and weight were measured at baseline and endline using a combined anthropometric scale and stadiometer (Seca, model no 887 7021094; serial no. 5877200147452, Hamburg, Germany). Childrens' ages were verified for 48% of participants by asking each mother/guardian to present the child's birth certificate or immunization card. Verbal recalls were obtained from parents who were unable to provide a formal proof of their children's' age. Anthropometric data were analysed with Anthro (World Health Organization (WHO) version 3.2.2.) and height-for-age (HAZ), weight-for-age (WAZ) and weight-for-height (WHZ) z-scores were computed.

**Definitions and data analysis**

Data analysis was conducted using STATA software (version 13). Non-normal variables such as CRP and serum β-carotene concentration were log-transformed. Serum CRP and AGP are commonly used to adjust for the effect of inflammation on serum retinol concentrations. However, it was recently shown that in a malaria-endemic population, CRP alone explained the highest percentage of the variance in serum retinol concentrations, in addition to the observation that AGP explained little of the remaining variance [30]. We thus used only CRP for inflammation adjustment as described by Palmer et al. [30]. Vitamin A deficiency was defined as serum retinol concentration <0.7μmol/L, after adjustment for CRP [31]. Moderate inflammation was defined as serum CRP >5mg/L and ≤ 15mg/L; high inflammation, serum CRP> 15mg/L [30]; anemia, serum Hb <110g/L; iron deficiency, serum ferritin concentration <30ug/L when children with inflammation were included, or <12 μg/L when children with inflammation were excluded [32]; serum soluble transferrin receptor concentration <8.3μg/ml [33]; malaria infection, plasmodium parasite count per microliter of whole blood>0; stunting, wasting and underweight were defined as ≤-2 z-scores of WHO child growth references for height-for-age, weight-for-height and weight-for-age respectively [34]. Feeding compliance was predefined as the ability to consume the minimum targeted amount of intervention food per day, i.e. ≥100g of eba, ≥32g of garri, ≥15g of *moinmoin*. Adequate vitamin A intake was defined as 210μg for children < 4 years and 275μg for children ≥ years[35].

Analysis of covariance (ANCOVA) was conducted to calculate the unbiased estimate of the average treatment effect [36]. In the crude analysis of the primary outcome (serum retinol), an adjustment was made only for randomization stratum, whereas, in the adjusted analysis, inflammation-adjusted baseline serum retinol concentration, C-reactive protein at endline and presence of malaria parasites were included as covariates, to adjust for the independent effects of inflammation and malaria on serum retinol concentration. Crude and adjusted models were built for other outcomes similarly by including appropriate variables.

**RESULTS**

Out of the 568 participants invited for the study, 77% (n=439) were screened, 46% (n=262) were selected for the run-in, and 31% (n=176) were eventually randomized, after meeting the baseline inclusion criteria. (**figure 5**). The study recorded a drop-out of 8% (n=14), mainly because participants' parents lost interest in the study. In total, data from 159 subjects were analysed, n=80 samples from the yellow cassava group, and n=79 from the white cassava group. At baseline, 23% (n=39) of the participants were stunted, 2% (n=4) wasted, and 13% (n=21) underweight

Inflammation biomarkers at baseline showed that 25% (n=44) of the children had elevated CRP (>5 mg/L) concentrations, and 5% (n=8) were infected with *Plasmodium falciparum*. However, none of the children presented with fever. After correction for inflammation, mean serum retinol concentration was 1.06 µmol/L, and 9% (n=16) of the children were vitamin A deficient at baseline. Iron deficiency was not found in the study population.

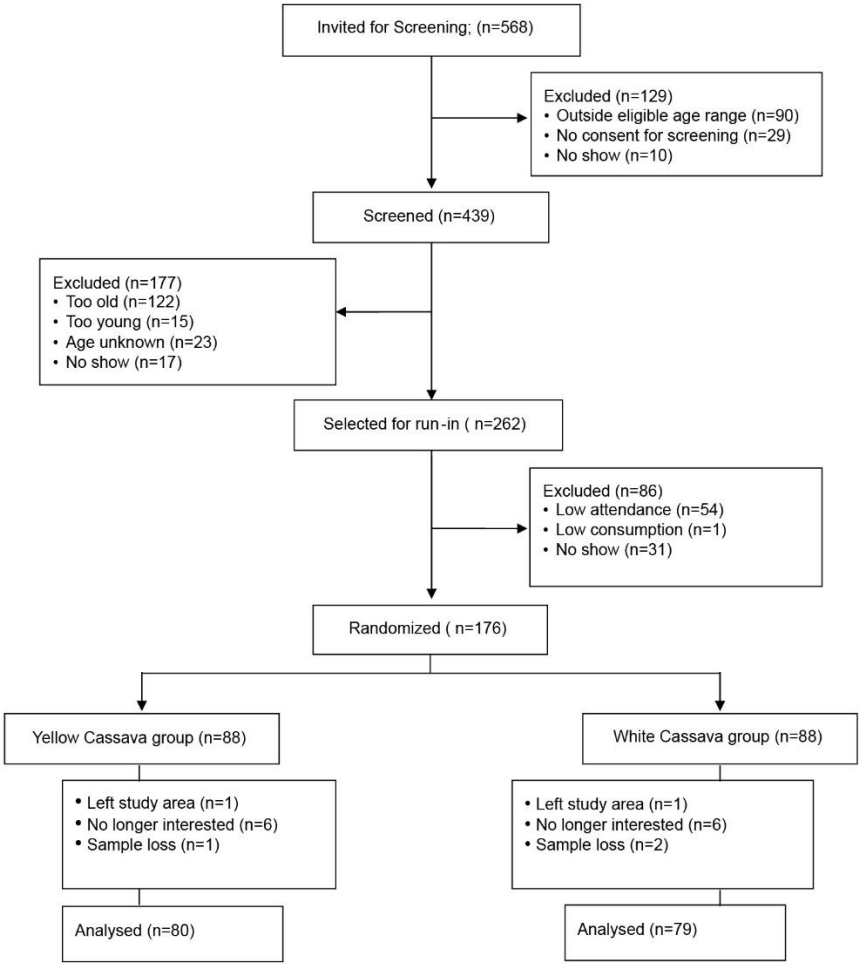


**Figure 5:** RCT flow diagram

**Table 2.** Baseline characteristics by intervention group

| Characteristics | White Cassava Group [n=88] | Yellow Cassava Group [n=88] | P-value* |
|---|---|---|---|
| Age (months)[1,2] | 49.2±10.9 | 49.1±12.4 | 0.95 |
| Male, n (%) | 51 (58) | 49 (56) | 0.85 |
| Female, n (%) | 37 (42) | 39 (44) | |
| Weight (kg)[2] | 14.5±1.97 | 14.4 ± 2.10 | 0.99 |
| Height (cm)[2] | 98.2 ±7.50 | 98.4±7.43 | 0.74 |
| Weight-for-height z-score[2,3] | -0.35±0.84 | -0.51±0.82 | 0.24 |
| Weight-for-age z-score[2,3] | -1.08±0.98 | -1.05±0.94 | 0.77 |
| Height-for-age z-score[2,3] | -1.36±1.32 | -1.18±1.29 | 0.44 |
| Height-for-age z-score <-2, n (%) | 23 (28) | 16 (18) | 0.93 |
| *Serum Inflammation Markers* | | | |
| $\alpha_1$-acid glycoprotein (AGP)[4], g/L | 0.9 (0.6, 1.4) | 0.9 (0.6, 1.5) | 0.65 |
| C-reactive Protein, mg/L[4] | 1.5 (0.5, 5.1) | 0.9 (0.4,5.1) | 0.61 |
| C-reactive protein (CRP) < 5mg/L, n (%) | 66 (75) | 66 (75) | 0.16 |
| Moderate inflammation n (%)[5] | 14 (16) | 11 (13) | 0.83 |
| High inflammation n (%)[6] | 8 (9) | 11 (12.5) | 0.80 |
| CRP >5mg/L or AGP >1g/L, n (%) | 40 (45.5) | 38 (43.2) | 0.88 |
| Malaria infection n (%) | 1(1) | 7(8) | |
| *Serum vitamin A markers (all children)* | | | |
| Retinol µmol/L[2] | 1.08± 0.26 | 1.04±0.26 | 0.27 |
| Vitamin A Deficiency (retinol <0.70 µmol/L), n (%) | 7 (8) | 9 (10) | 0.18 |
| β-carotene, µmol/L[2,4] | 1.82 (1.25, 2.48) | 1.90 (1.35, 2.42) | 0.21 |
| Retinol Binding Protein, µmol/L[4] | 0.96 (0.80, 1.15) | 0.94 (0.78, 1.15) | 0.76 |

| | | | |
|---|---|---|---|
| *Iron Markers* | | | |
| Hemoglobin, g/L | 116.9±11.5 | 117.4 ± 15.6 | 0.90 |
| Anemia, n (%)[7] | 23 (12.5) | 29 (16.5) | 0.47 |
| Serum ferritin (*all children*), µg/L | 86.4±34.2 | 92.8±36.4 | 0.39 |
| Iron deficiency, n (%)[8] | 0(0) | 0(0) | - |
| Serum ferritin (*children without inflammation*) | 83.5±28.7 | 79.2±26.7 | 0.24 |
| Iron deficiency, n (%)[8] | 0(0) | 0(0) | - |
| Serum soluble transferrin receptor, mg/L[6,8] | 7.9 (7.3, 8.5) | 8.7 (7.9, 9.4) | 0.10 |

*Statistical significance between groups was calculated using independent t-test; [1]Age was available for only 82 children in yellow cassava group and 86 children in the white cassava group; [2]Mean ± standard deviation; [3]WHZ, WAZ, and HAZ: In yellow cassava group, n=82; in white cassava group, n=86; [4]Median ($25^{th}$, $75^{th}$ percentile) [5]Moderate inflammation is defined as C-reactive protein concentrations >5mg/L and/ ≤ 15mg/L; [6]High inflammation is defined as serum CRP > 15mg/L; [7]Anemia is defined as Hb concentration <110g/L; [8]Iron deficiency is defined as serum ferritin concentration <30µg/L and soluble transferrin receptor concentration >8.3mg/L.

Participants consumed on average, 237g of *eba*, 32g of *garri*, 50g of *moinmoin*, and 65g vegetable soup daily in the yellow cassava group and 246g of *eba,* 35g of *garri*, 49g of *moinmoin and* 74g of vegetable soup daily in the white cassava group, **(table 3)** with an overall compliance of 98% in both groups. Children attended 87% of the intervention days, with no differences between white cassava and yellow cassava group; however, a small proportion of children (7%) attended less than 80% of intervention days. Within the pre-school, 221µg RAE was consumed in the yellow cassava group and 74µg RAE in the white cassava group per day **(table 3)**. The mean baseline serum retinol concentration (±SD) of children who had received vitamin A supplementation four months before the study was 0.95 ± 0.25 µmol/L, while children who did not receive vitamin A supplementation prior to the study had a mean serum retinol of 1.03 ± 0.02 µmol/L (p=0.16). The mean usual intake from foods consumed both in the pre-school and at home was 458µg RAE in the yellow cassava group and 292µg RAE in the white cassava group , and children less than four years had a mean retinol intake of 563µg and 293 µg in the yellow and white cassava groups respectively, while children four years and above had a mean retinol intake of 469µg and 303µg in the yellow and white cassava groups respectively. In addition, retinol inadequacy was 9% and 29% in the yellow cassava and white cassava groups respectively. Outside the preschool, palm oil contributed ~24% and 21% of RAE intakes in the yellow and white cassava groups respectively (p=0.6). There was no statistically significant difference in the adjusted mean RAE intake from foods consumed outside the pre-school between the yellow cassava and white cassava groups (p=0.46).

Unadjusted serum retinol concentration at endline was 0.99 ± 0.03 µmol/L and 0.96 ± 0.02 µmol/L for the yellow cassava and white cassava groups respectively **(table 4)**. At endline, 17% (n=28) of the children had elevated CRP concentrations (16 in the yellow cassava group and 12 in the white cassava group), and 33% (n=52) were infected with plasmodium parasites (32 in the yellow cassava group and 20 in the white cassava group), with the highest count reaching 57,109 parasites/µL. After adjustment for inflammation, malaria infection accounted for an additional reduction in serum retinol concentrations by 0.03 µmol/L in the entire study population. Vitamin A deficiency reduced to 8% (n=6 and n=7 in the yellow and white cassava groups respectively) but did not differ between groups (p=0.22). There was no correlation between RAE intake and adjusted serum retinol concentration (p=0.6)

The intervention resulted in a significant difference in serum retinol concentration between yellow and white cassava groups at endline **(table 4)**, with an effect size of 0.06 µmol/L (95% CI: 0.004, 0.124), after adjustment

for randomization stratum, inflammation, and malaria **(table 4)**. AGP was neither associated with serum retinol at baseline (exponential beta=0.03 [95% CI: -0.06, 0.11) nor at endline (exponential beta= -0.04 [95% CI: -0.12, 0.04). When AGP was included in the model, the effect of the intervention increased to 0.08 (p=0.012). A similar effect size was found for serum RBP concentration (effect: 0.08 μmol/L [95% CI: 0.01, 0.14]), whereas serum β-carotene concentration did not differ between treatment groups (effect: 3.8 % ; [95% CI: -0.6%, 8.6%]). No significant treatment effect was found on indicators of gut permeability. However, a significant increase in Hb concentrations was observed in the yellow cassava group [3.08 g/L (95% CI: 0.38, 5.78)] compared to the white cassava group. At endline, malaria infection alone explained 13% of the variation in Hb concentration and there was a 14% reduction in Hb in children with malaria (p=0.001). When excluding children with malaria parasites at endline (48 children remaining in the yellow cassava group and 57 in the white cassava group), treatment effects were 0.09 μmol/L (95% CI: 0.02, 0.16) for serum retinol and 3.10 g/L (95% CI: -0.32, 6.52) for Hb concentrations.

**Table 3.** Estimated daily intake of β-carotene and RAE[1] from intervention foods during the feeding trial

| | **White Cassava Group** n=79 | | | **Yellow cassava group** n=80 | | |
|---|---|---|---|---|---|---|
| | g/day | β-carotene, µg/day | RAE, µg/day | g/day | β-carotene, µg/day | RAE, µg/day |
| Cassava foods[2] | | | | | | |
| Eba | 246 (235, 256) | 36.9 (35.3, 38.4) | 5.3 (5.0, 5.5) | 237 (228, 246) | 760.8 (731.9, 789.7) | 108.57 (104.4, 112.8) |
| Garri | 35 (33, 37) | 18.9 (17.2, 20.15) | 2.7 (2.5, 2.9) | 32 (29, 34) | 305.3 (276.7, 324.4) | 43.6 (39.5, 46.3) |
| Moinmoin | 49 (45, 53) | 66.0 (60.3, 71.0) | 10.2 (8.6, 10.1) | 50 (45, 54) | 133.0 (119.7, 143.6) | 19.0 (17.1, 20.5) |
| Vegetable soups[2,3] | | | | | | |
| *Okra* | 62 (55, 69) | 251.4 (224.4, 278.4) | 35.9 (32.1, 39.8) | 55 (50, 59) | 221.1 (152.8, 239.0) | 31.6 (21.8, 34.1) |
| *Ewedu* | 12 (10, 13) | 145.5 (127.4, 161.5) | 20.8 (18.2, 23.1) | 10 (9,11) | 125.2 (115.4, 133.4) | 17.9 (16.5, 19.1) |
| Total | - | 518.7 | 74.1 | - | 1545.4 | 220.8 |

[1] RAE: Retinol Activity Equivalent; calculated based on ß-carotene:retinol conversion factor of 7:1 [23]; [2] Values are median (interquartile range); [3] The vegetable soup was a mixture of cooked okra+stew and cooked Ewedu+stew (See online supplementary methods for recipes).

**Table 4.** The treatment effect of consumption of yellow cassava on various outcomes[1]

| Outcome/intervention group | n | Estimated mean | Intervention Effect | |
|---|---|---|---|---|
| | | | Crude (95% CI) | Adjusted (95% CI) |
| Serum retinol concentration, µmol/L | | | | |
| White cassava group | 79 | 0.96 ± 0.02 | Reference | Reference |
| Yellow cassava group | 78 | 0.99 ± 0.03 | 0.032 (-0.042, 0.106)[2] | 0.06 (0.004, 0.124)[3] |
| Serum β-carotene concentration µmol/L | | | | |
| White cassava group | 79 | 2.51 (1.76, 3.36)[4] | Reference | Reference |
| Yellow cassava group | 78 | 2.64 (2.10, 3.57) | 3.3% (-1.2%, 7.8%)[2] | 3.9% (-0.6%, 8.6%)[5] |
| Serum RBP concentration µmol/L | | | | |
| White cassava group | 79 | 0.86 ± 0.03 | Reference | Reference |
| Yellow cassava group | 80 | 0.91 ± 0.03 | 0.06 (-0.01, 0.13) | 0.08 (0.02, 0.14)[6] |
| Haemoglobin concentration, g/L | | | | |
| White cassava group | 79 | 108.5 ± 9.9 | Reference | Reference |
| Yellow cassava group | 80 | 110.8 ± 10.0 | 2.32 (-5.45, 0.81)[2] | 3.08 (0.38, 5.78)[7] |
| Lactulose:mannitol ratio | | | | |
| White cassava group | 78 | 0.021 (0.018 , 0.024) | Reference | Reference |
| Yellow cassava group | 77 | 0.020 (0.017 , 0.023) | 0.008 (-0.086, 0.103) | 0.002 (-0.089, 0.092)[8] |

[1]Intervention groups were compared using ANCOVA; [2]Only adjusted for RBP stratum; [3]Additionally adjusted for inflammation-adjusted retinol at baseline and for C-reactive protein and malaria status at endline; [4]Values are median (25$^{th}$, 75$^{th}$ perecentile); [5]Aditionally adjusted for serum β-carotene at baseline and malaria at endline; [6]Aditionally adjusted for inflammation at baseline, retinol binding protein at baseline and malaria at endline; [7]Aditionally adjusted for Hb at baseline and malaria status at endline; [8]Additionally adjusted for urinary volume.

## DISCUSSION

Our results show that daily consumption of biofortified (yellow) cassava modestly prevented a decline in serum retinol concentration in a study population of Nigerian pre-schoolers, after adjusting for inflammation and malaria. The treatment effect was larger for children without malaria parasites in their blood circulation. In addition, we observed a small treatment effect on Hb concentrations. We did not find evidence for an improvement in gut permeability after exposure to the biofortified cassava foods.



The biggest constraint in the current study was our inability to assess body retinol pools as planned. We excluded a number of potential explanations to be the cause for this, such as: 1) verification of our analytical method by repeating LC-MS/MS analysis in the Lietz' laboratory at the University of Newcastle upon Tyne, UK [28]; 2) verification of the $^{13}$C-retinyl acetate dose by HPLC and LC-MS/MS analysis; and 3) strictly supervised capsule administration to our study participants. In addition, one of the authors took 2 capsules and had her blood taken 4 and 6 days later, showing clear isotope peaks in the plasma. Therefore, in retrospect, the administered dose of 0.4 mg may have been too low and the equilibration period of 14 days too long to be able to still assess the tracer in our study population. Recent studies suggest a shorter equilibration period of 3 – 6 days to be sufficient for predicting vitamin A stores reliably [37]. An additional explanation may be that absorption and retention of the dose was low due to the presence of inflammation in the study population as shown previously [38].

Much to our surprise, we did find a small but significant treatment effect on the modified primary study outcome: serum retinol concentration. This was unexpected, since we did not base our sample size on this outcome. In addition, serum retinol concentration is supposed to be rather irresponsive to intervention when vitamin A status of the population is in the sufficient range, and several colleagues in the field previously failed to show an effect of intervention on this outcome measure [2,3], whereas others did [4,39,40]. An important characteristic of studies that do report an effect of intervention on serum retinol concentration is that those studies adjusted their end of study values for baseline values. This is justified when the response to treatment depends heavily on starting values, as is the case with serum retinol. Correction for baseline values considerably spikes up the statistical power [41–43]. Another important difference with most other intervention studies is that we adjusted for inflammatory markers. The impact of inflammation on serum retinol concentration is quite established [30,44–46]. Helminth, bacterial and viral infections can trigger inflammatory mechanisms, which consequently lead to a decrease in negative acute-

phase proteins, such as RBP, responsible for the transportation of retinol from the liver to other body compartments [47,48]. As a result, the mobilization of retinol from the liver to other body compartments may be impaired. Infection-induced inflammation is thus an important confounder of serum retinol concentrations and masks true vitamin A status.

It has been postulated that the presence of malaria parasites lowers serum retinol concentrations independently of inflammatory processes [12,13]. In Nigeria, malaria is endemic with approximately 110 million clinically-diagnosed cases of malaria each year [49]. Therefore, we attempted to reduce the confounding effect of inflammation, by treating all children with deworming medication and antimalarials two weeks before baseline and endline assessment. However, we still detected *Plasmodium* parasites in 33% of the children at endline. In order to accommodate for this, we included the presence of malaria parasites as a covariate in the model in addition to C-reactive protein concentration. We found that presence of malaria parasites accounted for an additional drop in serum retinol concentrations of 0.03 μmol/L. A similar observation was made earlier in a study conducted in a malaria-endemic region in Burkina Faso, from which authors concluded that inflammation and malaria appeared to alter serum retinol concentration in a semi-independent manner [45]. The possible mechanisms that may explain this phenomenon include: (1) A reduction in RBP synthesis during malaria infection, in a manner independent of the acute phase response; (2) A parasite-induced impairment in the release of retinol from the liver; (3) an increase in the renal excretion of retinol due to malaria infection. Further studies are needed to understand the exact mechanisms responsible for this phenomenon.

Unexpectedly, we did not find any evidence of iron deficiency in the population even at baseline. In a more recent study conducted in a south west Nigeria, we equally did not find iron deficiency. (Owolabi *et al,* 2018; unpublished data). Previous studies have shown that provision of vitamin A may also increase iron indicators and Hb concentrations [50,51]. This may be explained by the role of vitamin A in stimulating erythropoiesis, thereby mobilizing iron to be incorporated into Hb [52]. However, in a malaria endemic region like Nigeria, the real effect of such interventions may be masked, due to the confounding effect of malaria on serum Hb: malaria infection initiates hemolysis of both parasitized and non-parasitized red blood cells which leads to a depletion of Hb concentrations, thus leading to anemia [53]. In the present study, we found a significant effect of yellow cassava on Hb, but only after adjusting for malaria infection.

We also observed that serum β-carotene concentration was very high at baseline (~2.0 μmol/L), and did not change in response to intervention. This lack of response to treatment may have been due to the two-week wash-

out period before endline assessments were conducted. During this period, children returned to consuming non-biofortified cassava foods and most of the β-carotene from the intervention available in systemic circulation may have been stored in the liver and/or extra-hepatic stores [54]. Nevertheless, serum β-carotene concentration was much higher than the 0.17 – 0.35 μmol/L reported from previous studies conducted in Kenya, Zambia, Bangladesh and Philippines [4,17,55,56]. For unknown reasons, serum β-carotene concentration seems to be high in Nigerian children, as previously reported by Adelekan *et al.* [56]. This may have to do with the regular intake of high doses of β-carotene from foods prepared with palm oil, which was consumed by over 80% of the children outside the intervention, contributing approximately 24% and 21% of the total RAE intake in the yellow and white cassava groups respectively. We speculate that regular intake of high doses of β-carotene at once may exceed the capacity to convert β-carotene to retinol in the enterocytes, thereby leading to high systemic concentrations of β-carotene. Alternatively, the specific population under study may be genetically predisposed as poor converters of β-carotene. These hypotheses require further investigation.

A major strength of this intervention was that the biofortified meals were prepared using the local recipes available in the community. This resulted in a varied and well-accepted menu for the children. Secondly, compliance to treatment and study procedures was high, due to the pre-school that we set up specifically for conduction of the trial. This enabled us to exercise a high level of control over the feeding sessions and all other procedures. Thirdly, we have been able to measure and adjust for major confounders of serum retinol concentrations yielding unbiased treatment effects.

The study was also limited by certain factors. Apart from the necessity to modify the primary outcome measure as outlined above, it was not possible to control for foods consumed by participants at home outside the intervention meals. However, considering the random allocation to treatment groups, RAE intake at home can be considered to be similar in both groups which was also reflected in the 24-hr dietary recalls, and is therefore not regarded as a confounder of much influence. A second limitation was the low prevalence of vitamin A deficiency in the population, which probably contributed to the small treatment effect. The Northern region of Nigeria would have been a better population for this study, based on the high reported prevalence of VAD [7]. However due to logistic and safety reasons, we could not conduct the study in that region. We also could not measure serum zinc as described in the study protocol because there was insufficient serum which was exhausted during the necessitated repeated analysis.

In summary, after adjusting for the effects of inflammation and malaria on serum retinol concentration, a modest treatment effect in serum retinol concentrations was found for daily consumption of biofortified (yellow) cassava. Consumption of yellow cassava can therefore be considered as an efficacious strategy to improve vitamin A concentration in cassava-consuming populations, and especially so in areas and population groups where vitamin A sufficiency is even more critical.

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The authors' responsibilities are as follows: IA coordinated the field work, conducted the statistical analysis and prepared the first draft of the manuscript, MM supervised the study and was involved in the field administration; FS provided general management and facilitated field logistics; PI supervised cassava production; EF assisted in data interpretation and analysis; ET contributed in preparing the first draft of the manuscript; AM-B oversaw all aspects of the study and carried overall responsibility; all authors contributed to the final manuscript.

EB and PI are employed by HarvestPlus; ET has previously been employed by HarvestPlus; Other authors do not have a conflict of interest. The funders had no role in the design, implementation, analysis or interpretation of the data.

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# Chapter Three

## Assessment of Small-Intestine Permeability in Healthy Nigerian Children is Altered by Urinary Volume and Voiding Status

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## ABSTRACT

### Objective:

This study aimed to uncover the effect of voided urinary volume on small intestine permeability ratios in healthy children.

### Methods:

We assessed small intestine permeability in 155 apparently healthy children, aged 3-5 years old, without any visible symptoms of disease, in a rural, malaria-endemic setting in Nigeria, using a multi-sugar test solution, comprising lactulose, sucrose, mannitol, and rhamnose. Children were categorized into low urinary volume (LV) and high urinary volume (HV), based on the volume of urine voided per kg body weight per hour. LV children voided less than 25$^{th}$ percentile of the total population, while HV children voided greater than 75th percentile of the total population. Urinary volume excreted over a 90-minute period after administration of the test solution was measured, and differences in sugar ratios were compared between children with high (HV) and low urinary volumes (LV), as well as between children who voided (VC) or who were not able to void (NVC) before administration of the test solution.

### Results:

Urinary mannitol and rhamnose recovery were 44% ($p=0.002$) and 77% ($p<0.001$) higher in HV children compared to LV children respectively, while urinary lactulose recovery was 34% lower ($p=0.071$). There was no difference in urinary sucrose recovery between groups ($p=0.74$). Lactulose-mannitol ratio, lactulose rhamnose ratio and sucrose-rhamnose ratio were all significantly higher in children in the LV group compared to children in the HV group ($p<0.001$). In a multiple regression analysis, urinary volume and voiding status combined, explained 13% and 23% and 7% of the variation observed in lactulose-mannitol, lactulose-rhamnose and sucrose-rhamnose ratios respectively.

### Conclusion

Sugar permeability ratios vary significantly with total urinary volume in multi-sugar small-intestine permeability tests. Voiding status before sugar administration appears to influence lactulose recovery, lactulose-rhamnose and sucrose-rhamnose ratios independently of total urinary volume. Evidence from this study suggests the need to take urinary volume into account when conducting multi-sugar small-intestine permeability tests.


**Keywords:** Gut permeability ratios, lactulose-mannitol test, Small-intestine permeability

## Introduction

Gut permeability is a terminology used to describe the control of material exchange between the lumen of the Gastro Intestinal Tract (GIT) and the rest of the internal body system. This control implies a physical barrier: comprising of the epithelial cell-lining of the gut, and a mucus layer; and a chemical barrier: comprising of digestive secretions, immune molecules, inflammatory mediators, and antimicrobial peptides [1]. Under certain conditions, such as during an infection or disease, the morphology and/or physiology of epithelial cells can be impaired, thus, leading to an abnormal permeability in the nutrient-absorbing region of the gut [2,3]. This phenomenon is observed in certain enteropathies and microbial infections and is often used as an indicator of gut integrity [4–7 ].

Gut integrity can be assessed by measuring the transcellular and paracellular transport of certain high and low-molecular-weight sugars across the nutrient-absorbing regions of the gastro intestinal tract [7]. These transport systems have been shown to follow diverse mechanisms amongst which include passive paracellular diffusion, active influx transport, active efflux transport and transcytosis [2,9,10]. Out of all the sugars studied, lactulose, rhamnose and mannitol have emerged to be the most common and most widely studied [11,12]. Traditionally, gut permeability is expressed as a ratio of the fractional excretion of a larger sugar molecule (e.g. lactulose) to that of a smaller one (e.g. mannitol or rhamnose). A higher urinary lactulose concentration thus reflects a higher permeability (paracellular transport), whereas, a lower urinary mannitol or rhamnose concentration reflects an impaired cell surface area (transcellular transport) [13].

Gut permeability assessment, using the lactulose-mannitol or lactulose-rhamnose test, therefore makes it relatively easy to determine gut integrity in a non-invasive manner. However, one major concern commonly reported using this test, is a high between-subject variation in sugar ratios [14,15]. Some of these variations appear to be methodological [14,16] Multiple conditions ranging from fluid loading [17,18] to severe diarrhoea [14] can reduce the volume of urine voided during gut permeability tests .These may also contribute to the variations in permeability ratios and lead to errors in the interpretation of gut permeability.

We specifically aimed at assessing the effect of voiding status and urine volume on small intestine permeability ratios. Based on evidence that lactulose and mannitol sugars can already be found in the colon within two-hours of dosage [15]. For the purpose of the present study, we specifically tested small-intestine permeability in healthy children using a multi-sugar permeability test with a ninety-minute urine collection protocol to eliminate any possibility of absorption from other regions of the gut outside the small

intestine. Specifically, our aim was to assess the effect of voiding status and urine volume on small-intestine permeability ratios.

## METHODS

### Study Design and Population

This study was part of an 18-week Randomized Control Trial (RCT), conducted between December 2015 to April, 2016 in Telemu, Ilemowu and Asamu communities, in Osun State, south-west Nigeria. The primary aim of the RCT was to establish a proof-of-principle on the efficacy of pro-vitamin A biofortified (yellow) cassava on the total body vitamin A pool in children 3-5 years old. Recruited children were enrolled in a pre-school, established for the purpose of the study and were divided into two groups: an experimental group (n=88 children), who fed on foods prepared with yellow cassava, and a control group (n=88), who fed on foods prepared with white cassava. Study ethical approval was obtained by the Ethical Review Board of Wageningen University (the Netherlands), University of Ibadan (Nigeria), and Osun State Ministry of Health (Nigeria). Parental consent was obtained before the commencement of the study during family visits and community meetings. All parents signed an informed-consent before their children were eligible to participate in the study. The study is registered on www.clinicaltrial.gov under the identification No. NCT02627222.

### Anthropometric Assessment

We measured participants' weight and height using a weighing scale and stadiometer (SECA model No. 887 7021094, Germany). Children's ages were verified by asking each mother/guardian to present the child's birth certificate or immunization card. The computed ages for 75 children, were based on verbal recall from their parent or guardian. Height for age z-scores (HAZ), and weight-for-height z-scores (WHZ) were computed using WHO Anthro (version 3.2.2).

### Small Intestine Permeability Tests

For the small-intestine permeability assessment, study participants were pooled from the two groups of the RCT. Twenty-one (n=21) children were excluded because they were either absent from school or were not fasted. One hundred and fifty-five (n=155) healthy children participated in the small intestine permeability test, which was conducted two weeks before the end of the RCT. All children were dewormed with a single dose (400mg) of albendazole and 300 mg prazinquantel at the beginning of the study, and were monitored for any form of sickness. Between the period of recruitment and small intestine permeability test, all the children who were diagnosed with malaria were treated, after which, they became eligible for

the test. Participants were therefore considered healthy before the commencement of small intestine permeability test.

The test solution contained lactulose (Sigma 61360; MM=342.30 g/mol), sucrose (Sigma S9378; MM=342.30 g/mol), D-mannitol (Sigma M4125; MM=182.17 g/mol), and L-Rhamnose monohydrate (Sigma R3875; MM=164.16 g/mol). Parents were reminded on the day before the test to ensure that their children came fasted. On the morning of the test, prescheduled children were assigned to small groups to ensure good supervision. Every child was carefully monitored by trained research assistants throughout the period of urine collection. Children were aided to void before sugar dosage, under supervision. Some children were unable to void, and these were therefore categorised separately during data analysis.



Forty grams each of lactulose and sucrose; and twenty grams each of mannitol and rhamnose, was weighed into a clean measuring beaker, and 1 litre of potable water was added. The solution was mixed by stirring with a clean spoon. After the dissolution of all the solids, the solution was aliquoted into 25mL medicine cups. Each child was administered this 25 mL portion containing 1.0 g each of the high molecular weight sugars (lactulose and sucrose) and 0.5 g each of the low molecular weight sugars (rhamnose and mannitol). This was followed immediately by giving 100-200 mL potable water to induce urine production. Sugar solutions were prepared, at most, ten minutes before dosage. Urine was collected within 90 minutes after sugar dosage into labelled disposable plastic containers, and the exact time interval between sugar dosage and urine collection was noted. Two drops of 20% chlorohexidine was added to urine samples to prevent bacterial degradation of the sugars. Urine weight and specific gravity were measured immediately after collection, using a weighing scale (Kern & Sohn, D-72336, Germany) and urinalysis strip (Surescreen diagnostics, Derby, UK). Urinary volume was calculated by dividing the mass by the specific gravity. Sample aliquots (5 mL) were stored at -20°C between the time of collection and analysis.

Urinary lactulose, mannitol, sucrose and rhamnose concentrations were measured by gas-liquid chromatography, as described by Jansen *et al*. (1986), using Tri-Sil-TBT derivatization, followed by separation on fused silica column, flame ionization detection and quantification by an internal standard method. All analyses were conducted in the laboratory of the Division of Human Nutrition and Health, Wageningen University and Research, the Netherlands. Internal controls were utilized according to the established laboratory protocol. All control samples run along the analysis were within ± 2 standard deviations.

**Calculations and Statistical Analyses**

The percentage recovery of each sugar in urine was calculated by multiplying the concentration (mg/ml) of the sugar in urine, by the total volume of urine collected over 90 minutes. Sugar recovery ratios were calculated by dividing the high molecular-weight sugars recovered in urine (i.e. lactulose or sucrose) by the low molecular weight sugars (i.e. mannitol or rhamnose). Data was entered using Stata version 13 (StataCorp, Texas, USA). To test for the effect of urinary volume on sugar recoveries, we created two contrasting categories of urinary volume, namely, low volume (LV) and high volume (HV). Low volume was defined as urinary volume per kilogram body weight per hour equal to or less than the twenty-fifth percentile of the population sample, while high volume was defined as urinary volume per kilogram body weight per hour equal to or greater than the seventy-fifth percentile of the same population sample. Children were also categorised into two groups: "voided children" (VC) and "non-voided children" (NVC), based on whether they voided or not before administering the test solutions. Group mean comparisons were analysed using independent t-test statistics. A multivariate regression analysis was also conducted to determine the independent effect of urinary volume, voiding status, urine specific gravity, HAZ, WAZ and WHZ on lactulose/mannitol or lactulose/rhamnose ratio. We controlled for the confounding effect of age, gender and intervention group a posteriori, by including these variables as covariates in the regression models. Percentage recoveries for lactulose, sucrose, mannitol, as well as lactulose-mannitol and lactulose-rhamnose ratios, were log-transformed for inferential statistical analyses. Geometric means, with their 95% confidence intervals are reported for all log-transformed variables, while arithmetic means are reported for non-transformed normally-distributed variables. All variables were tested for normality and skewness, using QQ plots, visual inspections, histogram and normality plots; and homoscedasticity, using the Levene's test. Statistical analyses was conducted using Stata (version 13) software.

**RESULTS**

The mean age of all the children who participated in the assessment was 53.6 months; their average height-for-age z-score was -1.00, while their weight-for-height z-score was -0.64 **(table 5)**. No significant difference was observed in height-for-age (HAZ), and weight-for-height (WHZ) z-scores between the LV and HV groups **(table 5)**.

**Table 5: Sugar Recovery and Recovery Ratios in Low and High Urinary Groups**

| | Low Urinary Volume (LV) (n=39) | High Urinary Volume (HV) (n=38) | All groups 95% CI (n=155) | Group Comparison |
|---|---|---|---|---|
| Mannitol Recovery [b] (%) | 3.62 [2.97, 4.39] | 5.19 [4.58, 5.89] | 4.76 [4.39, 5.15] | 1.44* [1.14, 1.81] |
| Rhamnose Recovery [a] (%) | 1.66 [1.39, 1.92] | 2.94 [2.55, 3.32] | 2.46 [2.28, 2.65] | 1.28* [0.82, 1.74] |
| Lactulose Recovery [b] (%) | 0.114 [0.92, 0.14] | 0.090 [0.07, 0.11] | 0.097 [0.087, 0.108] | 1.34 [0.54, 1.03] |
| Sucrose Recovery [b] (%) | 0.049 [0.036, 0.068] | 0.046 [0.037, 0.058] | 0.050 [0.044, 0.057] | 1.06 [0.72, 1.58] |
| Lactulose Mannitol Ratio [b] | 0.032 [0.027, 0.037] | 0.016 [0.013, 0.021] | 0.020 [0.018, 0.023] | 1.93* [1.43, 2.59] |
| Lactulose Rhamnose Ratio [b] | 0.081 [0.067, 0.098] | 0.031 [0.024, 0.041] | 0.045 [0.040, 0.051] | 2.58* [1.86, 3.56] |
| Sucrose Mannitol Ratio [b] | 0.014 [0.011, 0.017] | 0.009 [0.007, 0.012] | 0.010 [0.009, 0.012] | 1.51* [1.08, 2.11] |
| Sucrose Rhamnose Ratio [b] | 0.036 [0.027, 0.046] | 0.017 [0.013, 0.022] | 0.023 [0.020, 0.027] | 2.08* [1.45, 3.02] |
| Age (months) [a] | 53.6 [49.5, 57.6] | 53.9 [50.1, 57.8] | 53.6 [51.8, 55.3] | 0.38 [-5.8, 5.1] |
| HAZ [a] | -0.90 [-1.30, -0.50] | -1.01 [-1.47, -0.56] | -1.00 [-1.22, -0.77] | 0.12 [-0.47, 0.71] |
| WHZ [a] | -0.68 [-0.96, -0.40] | -0.73 [-1.05, -0.40] | -0.64 [-0.80, -0.48] | 0.05 [-0.37, 0.47] |

[a] Values are arithmetic means, and group comparisons are expressed as arithmetic differences, i.e. LV-HV or HV-LV as the case may be. [b] Values are geometric means, and group comparisons are expressed as arithmetic ratios i.e. LV/HV or HV/LV as the case may be.

**Table 6: Multivariate Linear Regression Analysis of the Association between Sugar Recovery Ratios and Urine Volume**

| Regression Model | Log (urine volume) $X_1$ | | | Voiding status $X_2$ | | | |
|---|---|---|---|---|---|---|---|
| | β | SE(β) | P-value | β | SE(β) | P-value | $R^2$ (adjusted) |
| Lactulose-mannitol ratio | -0.357 | 0.066 | 0.000 | -0.098 | 0.063 | 0.123 | 0.131 |
| Lactulose-rhamnose ratio | -0.487 | 0.071 | 0.000 | -0.206 | 0.068 | 0.003 * | 0.231 |
| Sucrose-mannitol ratio | -0.127 | 0.081 | 0.121 | -0.070 | 0.078 | 0.371 | 0.010 |
| Sucrose-rhamnose ratio | -0.269 | 0.088 | 0.003 | -0.177 | 0.084 | 0.037 * | 0.075 |

Model variables were adjusted for age and gender; * means model is significant, i.e. $p<0.05$.

**Sugar Recoveries and Small Intestine Permeability Ratios in LV and HV Groups**

Children who voided 4.92 ml of urine per kg body weight or more (HV group; void > 75$^{th}$ percentile), had higher recoveries of urinary mannitol (+44%, p=0.002) and rhamnose (+77%, p<0.002) compared to children who voided 1.28ml of urine per kg body weight or less (LV group, void> 25$^{th}$ percentile). At the same time, children in the HV group had lower recoveries of lactulose (-34%, p=0.071). Sucrose recovery was not statistically significant between groups p=0.74) **(table 5 and figure 6)**.



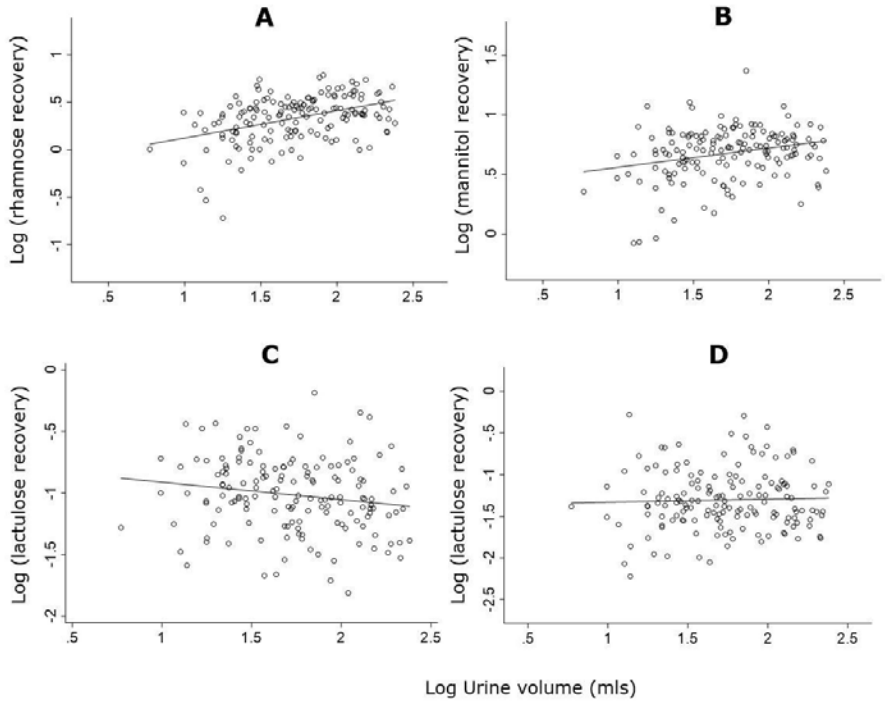


**Figure 6: Correlations between Sugar recoveries and Urine Volume:** A=rhamnose recovery (r=0.41 [p<0.001]); B=mannitol recovery (r=0.26 [p<0.001]); C=lactulose recovery (r=-0.16 [p=0.004]); D=sucrose recovery (r=0.035 {p=0.67]).

Sugar recovery ratios were all significantly higher in the LV group, compared to the HV group: lactulose-mannitol ratio (p<0.001), lactulose rhamnose ratio (p<0.001), sucrose-mannitol ratio (p=0.02), and sucrose-rhamnose ratio (p<0.001) **(table 5 and figure 7).**

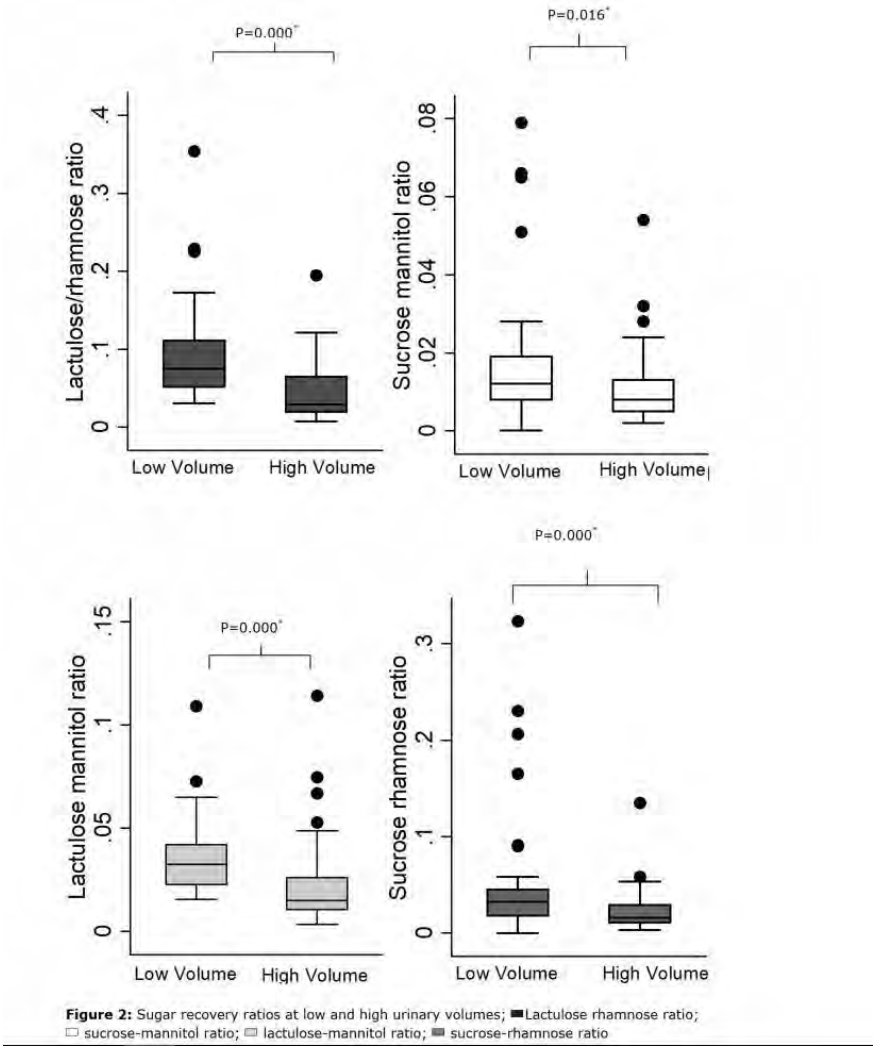


**Figure 2:** Sugar recovery ratios at low and high urinary volumes; ■Lactulose rhamnose ratio; □ sucrose-mannitol ratio; ▭ lactulose-mannitol ratio; ▬ sucrose-rhamnose ratio

**Figure 7:** Sugar recovery ratios at low and high urinary volumes; ■ Lactulose rhamnose ratio;□ sucrose-mannitol ratio; ▭ lactulose-mannitol ratio; ▬ sucrose-rhamnose ratio

**Comparison of Non-voided (NVC) and Voided (VC) Groups**

There was no difference in HAZ (p=0.6), WAZ (p=0.4), WHZ (p=0.3), age (p=0.07) and gender (p=0.3) between the NVC and VC groups Children in the NVC group (n=22) voided a mean urinary volume of 3.34ml/kg body weight/hour, equivalent to an average volume of 72.9 mL ± 54.2 mL of urine, whereas, children in the VC group (n=122) voided mean urinary volume of 3.28ml/kg body weight/hr, equivalent to an average volume of

72.4 mL ± 56.6 mL. (p=0.6). However, children in the NVC group had a significantly higher urinary density (1.0165g/L vs 1.0134 g/L, p=0.03). Furthermore, there was a 36% increment in lactulose recovery in the NVC group (p=0.05).

Regression analysis indicated that urinary volume and voiding status of children explained 13%, 23% and 7% of the variation in lactulose-mannitol ratio, lactulose-rhamnose ratio and sucrose-rhamnose ratio respectively. Also, a 1% increase in urinary volume significantly corresponded to a 0.3%, 0.4% and 0.2% decrease in lactulose-mannitol ratio, lactulose-rhamnose ratio, and sucrose-rhamnose ratio respectively **(table 6)**. Age and gender variables did not make any significant contribution to the model, however, they were retained in the model. Similarly, urine specific gravity, HAZ, and WHZ did not also make any contribution to the model, hence, these were removed from the regression model.



**Discussion**

In this study, we demonstrate that sugar recoveries and permeability ratios obtained during small intestine permeability assessments in relatively healthy children, are to a non-negligible extent, dependent on the volume of urine excreted during the sugar permeability test. In addition, the result of the test depends on whether the child was able to void before commencement of the test or not. This study therefore clearly suggests that the assessment of small intestinal permeability is dependent on volume of urine voided.

Our study reproduces similar findings reported previously in a five-hour gut permeability assessment for rhamnose recovery [3] and mannitol recovery [19], although we used a ninety-minute small intestine permeability assessment. In these studies, urinary volume was shown to contribute significantly to the variation in sugar permeability ratios. Rhamnose recovery was generally lower than mannitol in equal volumes of urine, despite similar doses of the two sugars were administered. This pattern has been reported in previous studies involving multi-sugar assessment, [20,21], however, it is not very clear whether this is attributable to membrane transport mechanisms (i.e. active transport versus passive diffusion across gut membrane) or competition for membrane transporters by other sugar solutes present in the solution. Menzies et al, (1990) [20] observed that in a multi-sugar test, involving lactulose, L-rhamnose, and mannitol, mannitol reduced intestinal uptake of rhamnose and lactulose [20].

Furthermore, we observed a different relationship between voiding status and percentage recoveries for lactulose sugar, which appears to be independent of excreted urinary volume. Even though the total urinary

volume produced by both voided and non-voided groups was not different, there was a 36% increment in lactulose recovery in the non-voided group. This could possibly reflect more concentrated urine due to a lower hydration state in this group, especially because the group also had a slightly higher urine specific gravity. However, this does not explain why only lactulose recovery was higher whereas recoveries of the other sugars did not differ from the group who voided before the test.

Our study does not answer the question why differences in urinary volume and voiding status result in differences in sugar recovery. Hence, it is not clear if LV and NVC truly had poorer gut function, or if the higher sugar recovery ratios rather result from other biologic conditions. Possible explanations are: 1) Children with lower urinary volume or those who could not void before the test may have been in a poorer state of hydration; 2) differences in renal clearance and/or water resorption rate between children; 3) a combination of both. There were however no obvious differences in age, sex or malnutrition status between the LV and HV groups, nor between VC and NVC groups. They all lived in the same village and underwent the same screening procedure for eligibility to participate in the study. Since they were enrolled in an RCT, they largely ate the same food and had the same activity pattern. We did not assess hydration status of the children, although we did see a difference in urinary specific gravity between VC and NVC groups. It may be that children with poorer gut function coincidently were at the same time more often dehydrated, in which case their higher sugar recovery ratios truly reflect poorer gut function.

There is conflicting data in previous studies on the association between chronic and acute forms of malnutrition and gut permeability. Some studies have reported associations [22–25], while others show no association at all [26,27]. In the present study, we found no association between small intestine permeability and height-for-age, weight-for-height and weight-for-age z-scores.

The mean values obtained for lactulose-mannitol and lactulose-rhamnose ratios from this study, compare very well with previous studies. Mean lactulose-mannitol, lactulose-rhamnose and sucrose-rhamnose ratios from this study were 0.020, 0.045 and 0.023 respectively, which falls within the range reported for healthy controls in non-hospital-based studies from African countries [28]. Additionally, we reviewed some relevant studies on gut permeability ratios conducted between 1979 and 2014 in Africa [22,28–30], United Kingdom [31], Europe [3], South America [14,32], Asia [24,27,33–35], and Australia [26]. In this, we made a distinction between hospital-based and non-hospital-based studies in order to differentiate gut permeability ratios reported in sick children from those reported in healthy

children across different regions. Reported values for healthy children ranged from 0.030 to 0.054 for lactulose-mannitol ratio, and 0.015 to 0.070 for lactulose-rhamnose ratio. Brewster *et al.* (1997) [28] observed a higher range in African healthy children (lactulose rhamnose ratio, 0.034–0.096) compared to children in Europe (lactulose rhamnose ratio, 0.027). In this context, our results appear to fall somewhere at the lower limit, despite the fact that being a rural setting, infection rates were supposedly quite high amongst participants. A reasonable explanation for this is the deworming regimen provided at the beginning of the study.

An important limitation of the study was our inability to test the likely possibility that the total duration of urine collection influenced sugar recovery or gut permeability ratios [36]. However, it most likely would not have changed our results significantly, because the total volume of urine collected in the two study groups was almost the same. Secondly, this study, being part of a randomized controlled trial on biofortified cassava, was not originally designed as a stand-alone study. We therefore could not measure baseline gut permeability, which would have provided additional time points for comparison.

In conclusion, data from this study strongly suggests that the volume of urine voided in children is a significant factor contributing to variations in small intestine permeability ratios, using sugar probes. We therefore recommend that urinary volume and voiding status be carefully monitored during gut permeability tests.

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# Chapter Four

## The Contribution of Pro-Vitamin A Biofortified Cassava to Vitamin A Intake in Nigerian Pre-school Children

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**ABSTRACT**

**Background**: Biofortified yellow cassava has been developed to alleviate vitamin A deficiency. We examined the potential contribution of yellow cassava to total Retinol Activity Equivalent (RAE) intake if replacing white by yellow cassava among pre-school Nigerian children.

**Methods:** Dietary intake was assessed as part of a randomized controlled trial. Preschool children (n=176) were randomly assigned to receive either white cassava (WC) or yellow cassava (YC) for 17 weeks. Dietary intake assessments were conducted during the intervention and one month after, when children had resumed their habitual diet. Differences in RAE intake between groups and time points were compared using a linear mixed model regression analysis.

**Results:** During intervention, median RAE intake was 536 µg/day in the YC group and 301 µg/day in the WC group ($p<0.0001$). YC contributed ~40% to total RAE intake and 63% of EAR. Nine percent of children in the YC versus 29% in the WC groups had RAE intake below the Estimated Average Requirement. After intervention, median RAE intake was 300 µg/day and did not differ between intervention groups ($p=0.5$). The interaction effect of group and time showed a 37% decrease in RAE intake in the YC group after the intervention ($Exp(\beta) = 0.63$ [95% CI 0.56, 0.72]). If WC was replaced by YC after intervention, the potential contribution of YC to total RAE intake was estimated to be ~32% .

**Conclusions:** Yellow cassava increased total RAE intake and showed a substantially lower inadequacy of intake. It is therefore recommended as a good source of provitamin A in cassava-consuming regions.

## INTRODUCTION

Vitamin A deficiency (VAD) is still a public health problem in many low-to-middle income countries (LMICs), affecting approximately 190 million pre-school aged children, which corresponds to about 33% of the children in this age group globally [1]. In 2013, the highest prevalence of VAD, was in south-Asia and sub-Saharan Africa with a prevalence of 44% and 48% respectively [2]. VAD impairs various physiological functions, and as a result, can pose serious health challenges to infants, children and pregnant women [3]. Approximately 94,500 deaths from diarrhoea and 11,200 deaths from measles worldwide were attributed to VAD in 2013, which accounted for 1.7% of all deaths in children under five years in LMICs. [1]

Nigeria is among the countries with the highest prevalence of VAD in Africa. Based on the available data from the only nationally representative food consumption and nutrient survey (2004), 29.5% of children under-five years old were classified as vitamin A deficient, with serum retinol concentrations below 0.7μmol/L [4], which is attributed to inadequate dietary intake. This high prevalence, which was regarded as a public health problem, led to the implementation of several strategies such as vitamin A supplementation (VAS) and food fortification to reduce VAD prevalence [5]. Over the years, the coverage of VAS supplementation has increased in many LMICs including Nigeria, however, the impact of VAS on the reduction of VAD has been at a very slow rate [6].

A food-based approach is a more sustainable approach to attaining micronutrient adequacy compared with other fortification approaches [7]. Biofortification has emerged as a complementary strategy to meet the micronutrient needs of vulnerable populations [8]. Specifically, several staples from sub-Saharan Africa and south-Asia have been biofortified with provitamin A, with the aim of complementing other vitamin A interventions [9]. Two randomized controlled trials have demonstrated the efficacy of pro-vitamin A biofortified crops in increasing serum retinol[10] and total body retinol pools [11,12] in children. Yellow cassava, a biofortified variety of the traditional white cassava, is a root and tuber crop, largely consumed in Nigeria. There are currently six varieties of yellow cassava available in Nigeria. These varieties are generally resistant to many pests and diseases, have high yields, and can produce up to 15 micrograms/gram of β-carotene [13]. However, till now, no study has considered the contribution of yellow cassava to the usual intake of vitamin A in a natural setting, outside an experimental study set-up.

This study therefore aimed at quantifying the contribution of biofortified (yellow) cassava to vitamin A intake of preschool children in a rural

Nigerian community where cassava is widely consumed as a common staple, both under experimental and free-living conditions.

## SUBJECTS AND METHODS

### Study Design and Participants

Dietary assessment was conducted in 2016 as part of a randomized controlled trial (RCT) aimed at assessing the efficacy of biofortified cassava varieties on serum vitamin A concentrations in pre-school children, aged 3 – 5 years old in three adjacent communities: *Telemu*, *Ilemowu* and *Asamu* Osun state, Nigeria. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the University of Ibadan/University College Hospital Ethical Review Committee (ERC), as well as a positive advice from the Medical Research Ethics Committee, Wageningen University and Research (WUR), Wageningen, the Netherlands. State Government approval was also obtained from the Osun State Ministry of Health. The study is registered in clinicaltrial.gov (ID NCT02627222). One hundred and seventy-six children were recruited into the RCT including dietary assessments. At least one participant was recruited from the twenty compounds identified in the communities, based on the following criteria: (1) their willingness to participate; (2) No visible sign of sickness; (3) older than 3 years and less than 5 years before study commencement; (4) plasma haemoglobin > =70g/L; and (5) ability to consume >=80% of the pre-established age-specific target meal portion for the RCT. Exclusion criteria included (1) severe anaemia (<7.0g/dl)/ symptomatic malaria or infectious diseases; (2) unwillingness to participate or no informed consent; (3) Absenteeism (i.e. not meeting up to 20% of feeding sessions); (4) Inability to consume the required amount of cassava (i.e ≥ 80% of the age-specified amount); (5) History of food allergy.

A pre-school was established specifically for the purpose of the intervention, where students were taught and fed between 8.00am and 2.00pm. Children whose parents/guardian had signed a written informed consent and who met the conditions for the inclusion criteria were registered in the pre-school, where they were fed breakfast and lunch, six days a week, for a period of 17 weeks. The participants were divided into two groups: the experimental group (n=88) and the control group (n=88). The experimental group consumed foods prepared with yellow cassava, (yellow cassava *eba,* yellow cassava *garri,* yellow cassava *moinmoin,* complemented with *okra* or *ewedu* soup), while the control group consumed the same foods prepared with white cassava. Children in the experimental and control groups were physically separated to prevent crossing-over. The children resumed school at 8.00 am, Standardized

recipes were developed for all foods consumed in the pre-school before the commencement of the intervention.

**Dietary intake assessments**

No dietary assessment was conducted at baseline. The first round of dietary assessment was conducted in the 15th week of the study period (n=162) to estimate dietary intake of the experimental and control groups both within the pre-school as well as at home. A second round of dietary assessment (n=158) was conducted one month after the intervention, to measure the intake of the study population after they returned to their habitual diets. In each round, repeated recalls were conducted for approximately 30% (n=45) of the children, to account for day-to-day variation and compute adjusted nutrient intake.

Within the pre-school, intervention foods were prepared in a central kitchen by trained cooks. All the ingredients used in cooking were weighed before cooking. Weights and total volume of dishes cooked were recorded daily and entered into a database. In the pre-school, trained research assistants weighed individual food portions before serving, using a 0.1 precision scale (Kern EMB 5.2K1, Germany). The same scales were used throughout the study period. All plates and cups were properly labelled with each participant's ID number. After the meal, left-overs were recorded and entered the same day into a central database.



Dietary intake for foods consumed outside the preschool was assessed using the quantitative interactive multi-pass 24-hour recall (24hR) method [14,15] on evenly-divided days over the week. Repeated recalls were conducted on non-consecutive days and distributed equally across the days of the week. Participants for repeated recalls were randomly selected from both experimental and control groups. Participants' dietary consumption was provided by parents (mother or father) or caregivers, who were directly responsible for children's diets. Dietary assessment was conducted at participant's residence by local research assistants, who spoke the local language and had been trained shortly before the time of the dietary assessment.

During the interview, each parent/caregiver was asked to mention all foods and beverages consumed by their child (from waking up) on the day before the interview, until waking up on the interview day. Detailed information on food ingredients, including preparation/processing methods, total amount prepared, portion consumed by the child and amount of left-overs were also collected during the recall session. Parents/caregivers were asked to demonstrate the procedures followed while preparing the recalled meals, using when possible, the same ingredients and household utensils (i.e.

cups, bowls and spoons). From these demonstrations, the amount of each ingredient in each meal consumed was estimated by direct weighing on a digital scale (Kern EMB 5.2K1, Germany).

**Conversion factors**

In households where the ingredients used for cooking were not available at the time of the recall, the volume or weight of comparable ingredients with similar texture, were used as alternatives (i.e. *proxy ingredients*) during the demonstration, to estimate amount consumed (for example, yam flour as an alternative to cassava flour, garri as an alternative to rice etc). After the recall, the actual ingredients were purchased from the community markets, and conversion factors (i.e. weight-to-weight; or volume-to-weight) were computed, to calculate the actual amount of the recalled ingredient. In some households, some of the recalled meals were bought as ready-to-eat dishes from local food vendors. In other households, some of the ingredients used to prepare a particular meal were purchased from the community markets. In both cases, price-to-weight conversion factors were used to estimate the amount of ingredients consumed.

Most of the dishes reported during the recall session were prepared for the entire household, from which the child consumed only a portion. We therefore estimated the total volume of dish cooked and the proportion consumed by the child using the method described by Gibson & Ferguson [14]. Market surveys were conducted during and after the intervention to obtain all conversion factors. The market survey was conducted within three weeks after each 24-hour recall data collection, to avoid the effect of price fluctuations on conversion factors. During the market surveys, food items or ingredients were purchased in the local currency (naira), from three different shops or vendors where community members frequently purchased their foods. The food items or ingredients were subsequently weighed with and without waste, and then averaged. Price-to-weight conversion factors were generated for the edible portion of the food or ingredient, by calculating the amount in the local currency corresponding to 1g of the food or ingredient. Weight-to-weight conversion factors were generated by calculating the amount of *proxy ingredient* corresponding to 1g of actual ingredient. Volume-to-weight-conversion factors were generated by calculating the volume of liquid/semi-solid food or ingredient corresponding to 1g of the food or ingredient. During recall, it was noted that some dishes were prepared by combining various food items for a total price, for instance, 100g rice + 50g beans + 20g stew=N50. In such cases, the proportion of each ingredient within the dish was calculated, so that the weight of each food item could be retrieved separately.

**Standard Recipes**

During the 24-hour dietary recall, the details of some food ingredients consumed by children could not be obtained because the dishes were bought from a food vendor, or the respondent could not remember the ingredients in the dish. To address these issues, standard recipes were developed to estimate the amount of the ingredient consumed. To develop the standard recipes, three randomly-selected volunteer mothers, whose children were participating in the study, were invited and asked to cook the specific dishes, using the same cooking methods employed at home. The different ingredients used in the preparation of the dishes were then recorded and weighed by research assistants while cooking was ongoing. Similarly, standard recipes were collected from random stalls and vendors within the community. In all cases, the edible portions of cleaned ingredients and the total volume of dish cooked were noted.

**Carotenoid analysis of food samples**

During the entire study , samples of intervention meals, as consumed, were randomly collected twice weekly for 17 weeks, into opaque containers and homogenized with 5ml butylhydroquinone (a preservative). Food samples were subsequently stored at -20°C at the field site and later shipped to WUR, where samples were stored at -80°C until analysis. During analysis, the food samples were pooled together as composite samples, representing three different intervention periods. Total β-carotene in food samples were analysed in duplicate using the HPLC (Thermo Scientific Accela LC system; Thermo Fisher Scientific) and EZCHrom Elite version 3.2.2 SP2 software (Argilent Technologies, Santa Clara, United States). The details of the extraction and HPLC analysis have been described elsewhere (Afolami *et al,* 2020).



**β-carotene retention in commonly consumed palm oil soups**

β-carotene retention in two commonly consumed palm oil-based soups was calculated experimentally. Through the careful observation of volunteer mothers invited to cook these soups, a recipe detailing the average amount of all ingredients in the soup was developed and standardized, taking note of the average cooking time and bleaching temperatures for palm oil. The total β-carotene content of all raw ingredients was then calculated using a food composition table [16], by summing the β-carotene content of individual raw ingredients, including raw palm oil, which was obtained from the same community. In addition, the β-carotene content of raw palm-oil was analysed. Using the standardized recipes, dishes of stew and *egusi* soups were prepared experimentally using either raw palm oil or standard vegetable oil as ingredient. The quantity and type of all ingredients in the analysed soups were identical and the only difference was the substitution of red palm oil with vegetable oil. Yield factors were calculated for both

soups immediately after cooking, and the soups were analysed for total β-carotene content in the form normally consumed. The true retention factor of β-carotene from the palm-oil soups was estimated using the formula:

$$True\ retention\ of\ \beta car = \frac{([\beta car]_{palm\ oil-cooked} - [\beta car]_{veg\ oil-cooked})}{([\beta car]_{palm\ oil-raw})}$$

where $[ßcar]_{palm\ oil\text{-}cooked}$ = β-carotene content per gram of cooked soup with palm oil; $[ßcar]_{veg\ oil\text{-}cooked}$ = β-carotene content per gram of cooked soup with regular vegetable oil; $[ßcar]_{palm\ oil\text{-}raw}$ = β-carotene content per gram of palm oil in raw soup; and the yield factor = total weight of cooked soup in grams divided by the weight of total raw soup ingredients in grams.

**Nutrient Intake Estimation**

To estimate nutrient intakes, a food composition table (FCT) was compiled for this study based on the Nutrient Composition of Commonly Eaten Foods in Nigeria- raw, processed and prepared [17]. The FCT was supplemented with nutrient composition from foods in other food composition tables in descending order of priority: West African Food Composition Table [16]; Condensed Food Composition Table for South Africa [18]; Food composition table for use in Ghana (1992). [16], United States Department of Agriculture food composition [19]; National Nutrient Database for Standard Reference, release 28 [20]; International Minilist [21].

In the absence of nutrient information for particular foods, nutrient composition was obtained from nutrition labels (for packaged foods) or from published scientific literature. A bioequivalence factor of 7:1 [22] was applied to convert β-carotene to retinol activity equivalent from cassava. For all other foods, a bioequivalence factor of 12:1 was applied [23]. Appropriate retention factors were applied to adjust for nutrient loss during preparation [19]. Compl-Eat software (version 1.0, WUR) was used in the overall computation of nutrient intake [24]. Vitamin A intake was expressed as Retinol Activity Equivalent (RAE) in µg/child/day. The average RAE intake was compared with the Estimated Average Requirement (EAR) to determine the adequacy of vitamin A intake using age-specific cut-off points provided by the Institute of Medicine (IOM) [23]. Estimations of RAE and percentage below EAR during the second round of dietary intake assessment was simulated, based on the assumption that the white cassava foods consumed during this time were replaced by yellow cassava foods. Total energy intake was expressed as kcal/child/day. Total energy intake was assessed by comparing total daily energy with the Estimated Energy Requirement (EER) as established by IOM, which considers the gender, age, height, weight and physical activity levels. Based on the observation of the daily routine of the children, a physical activity level of 1.16 (low-active

physical activity level) was used for all children to estimate their energy requirement [23].

**Anthropometric Measurement**

Height and weight measurements were collected at baseline and endline, using a combined anthropometric 0.2 precision scale and stadiometer (Seca model no 887 7021094; Hamburg, Germany). Anthropometric parameters (height-for-age (HAZ), weight-for-age (WAZ), and weight-for-height (WHZ) z-scores, respectively were analysed with Anthro (WHO version 3.2.2.).

**Statistical Analysis and Sample size**

The statistical analysis was carried out using Stata 13.0. The distribution of all variables used in the analyses were inspected for normality by combining QQ plots and visual inspections. Non-normal variables were log-transformed. All outliers were retained in the data because none of the estimated intake was considered implausible. Repeated recalls during each round of dietary intake assessment were analysed using ANOVA to estimate the magnitude of the within-person-variance and to adjust for day-to-day variation. Following this procedure, adjusted intake distributions were calculated for RAE and energy intake, using the method described by the National Research Council (NRC) [25] . A linear mixed regression method was used to estimate the difference (exp β) in RAE intake between the experimental and control groups based on the first and second rounds of dietary intake assessments. This model is advantageous because of its robustness in handling within-subject correlations resulting from repeated measurements. In fitting the model, log-transformed vitamin A intake was treated as the response variable, and the intervention group was included as a fixed effect. Correlation between the two-time points of dietary intake assessment was accounted for, by including the random-intercept of the subject (ID) in the model. Unstructured covariance with Restricted Maximum Likelihood estimation were used to estimate the variance component. Tukey-Kramer was applied to make multiple comparisons (interactions) of least square means among groups and time points. We adjusted for the confounding effect of age, gender and total energy intake by including these variables in the models. The best model was chosen by comparing the lowest Akaike information criterion. Wilcoxon non-parametric statistic was used to test for the difference in median energy and RAE between participants of different age categories.

Based on the projected daily intake of yellow and white cassava, we expected to detect a difference of at least 200 µg in the mean RAE daily intake between the yellow and white cassava groups. To this effect, a minimum sample size of 112 participants (56 per group) was required,

assuming a 15% attrition rate, an alpha and beta risk of 0.05 and 0.20 respectively. This was based on a standard deviation of 318 µg as reported in a study conducted in the same region [26]

## RESULTS

### Characteristics of the Study Population

One hundred and sixty-two children participated in the first round of the dietary intake assessment **(table 7)**, while 158 children participated in the second round **(table 8)**. The mean age of children was 4.1 ± 1.0 years. Fifty eight percent (n=94) of the children were males **(table 7).**

### Energy Intake and Vitamin A Intake

The energy and vitamin A intakes during and after the intervention (i.e. in the first and second rounds of dietary intake assessment) are presented in **table 8**. In the first round of dietary intake assessment, overall median energy intake was 1584 kcal/day, while in the second round, median energy intake was 1451 kcal/day ($p<0.001$). There was no difference in energy intake between boys and girls ($p=0.24$). Over 80% of the children had adequate energy intake during and after the intervention.

During the intervention, median RAE intake was 536 µg/day and 301 µg/day in the yellow cassava and white cassava groups, respectively ($p<0.0001$). However, after the intervention, median RAE intake decreased to 257 µg/day in the yellow cassava group and increased to 353 µg/day in the white cassava groups, respectively ($p=0.5$). In total, 82% of the children had adequate RAE intake during the intervention, which declined to 56% after the intervention ($p<0.0001$) **(table 8)**. There was no significant difference in RAE intake between boys and girls during and after the intervention ($p=0.58$ and $p=0.13$ respectively). During the intervention, yellow cassava contributed ~40% to the total RAE intake and 63% of EAR. The percentage of children with retinol activity intakes below the EAR was 9% (n=7) and 29% (n=23) in the yellow and white cassava groups, respectively. After the intervention, the percentage of children with retinol intakes below the EAR increased to 43%. Based on the assumption that consumed white cassava foods were all replaced with yellow cassava, the projected contribution to total RAE intake after intervention was estimated to be ~32% **(table 8).** Palm oil was the second largest contributor to vitamin A intake. During the intervention, palm oil contributed to ~24% and 21% of RAE intakes in the yellow and white cassava groups respectively ($p=0.6$). After the intervention, the contribution of palm oil to RAE intake was approximately 35%.

.

**Table 7**. Characteristics of study participants

| | | During Intervention | | After Intervention | |
|---|---|---|---|---|---|
| **Characteristic**: | All children (n=162) | Yellow cassava n= 82 | White cassava n=80 | Yellow cassava n=78 | White cassava n=80 |
| Female n (%) | 68(42) | 35 (43) | 33 (41) | 33 (42) | 34 (43) |
| Age ± SD | 4.1±1.0 | 4.1 ± 1.03 | 4.1±0.90 | 4.4±1.07 | 4.5 (1.0) |
| Weight (kg) | 14.38±2.06 | 14.38 ± 2.13 | 14.38±2.0 | 14.9 ± 2.11 | 14.8 ± 1.93 |
| Height (cm) | 98.17±7.56 | 97.97 ± 7.65 | 98.36±7.52 | 101 ± 7.3 | 101 ± 7.7 |
| Weight-for-height z score | -0.44±0.84 | -0.52 ± 0.84 | -0.35±0.83 | -0.72 0.80 | -0.57 ± 0.96 |
| Weight-for-age- z-score | -1.08±0.96 | -1.05 ± 0.93 | -1.10±0.99 | -1.12 ±0.93 | -1.21 ± 0.94 |
| Height-for-age z-score | -1.29±1.31 | -1.19 ± 1.26 | -1.39±1.37 | -1.08 ± 1.24 | -1.28 ± 1.36 |

**Table 8**. Energy and vitamin A intake during and after the intervention

| Variables | During Intervention | | After Intervention | | | Projected intake[1] |
|---|---|---|---|---|---|---|
| | Yellow cassava | White Cassava | Yellow Cassava | White Cassava | Total (n=158) | |
| **Energy intake[2]** | | | | | | |
| Low[3], n (%) | 20 (24) | 11 (14) | 16 (21) | 12 (15) | 28 (18) | |
| <4 years, kcal/d | 1506 (1322-1862)[a] | 1556 (1239-1845)[a] | 1431 (1381-1525)[a] | 1435 (1394-1498)[b] | 1434 (1381-1525) | |
| ≥ 4 years, kcal/d | 1560 (1298-1678)[a] | 1657 (1436-1794)[a] | 1470 (1357-1521)[a] | 1459 (1403-1523)[b] | 1461 (1392-1523) | |
| Girls, kcal/d | 1553 (1315-1685)[a] | 1657 (1470-1845)[a] | 1440 (1395-1565)[a] | 1453 (1430-1508)[a] | 1453 (1405-1537) | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Boys, kcal/d | 1526 (1311-1706)[a] | 1575 (1397-1793)[a] | 1452 (1351-1507)[a] | 1451 (1378-1523)[a] | 1452 (1353-1509) | |
| **RAE intake[2]** | | | | | | |
| Inadequacy n (%) | 7 (9) | 23 (29) | 38 (49) | 32 (40) | 70 (44) | |
| < 4 years, µg/d | 563 (374-623)[a] | 293 (260-392)[b] | 249 (178-649)[b] | 370 (170-622)[b] | 307 (177-625) | 475 (313-626) |
| ≥ 4 years, µg/d | 469 (328-586)[a] | 303 (246-388)[b] | 263 (184-532)[b] | 350 (186-526)[b] | 292 (186-526) | 561 (358-1017) |
| Girls, µg/d | 533 (308-606)[a] | 310 (260-462)[b] | 261 (198-528)[b] | 397 (223-698)[b] | 373 (198-584) | 531 (383-922) |
| Boys, µg/d | 542 (350-614)[a] | 288 (251-363)[b] | 254 (178-555)[b] | 300 (162-450)[b] | 273 (163-480) | 475 (318-743) |

RAE, retinol activity equivalents [1]Calculated from post-intervention data at time point 2 based on the assumption that yellow cassava foods replaced all white cassava foods for all children;
[2]Energy intake and RAE intake values are median (25$^{th}$, 75$^{th}$ percentile) unless stated otherwise;
Low energy intake is defined as energy intake less than IOM's recommended age-specific requirement
[a,b]Rows with different letters are statistically significantly different. Row letters compare both yellow and white cassava groups during and after the intervention.

**Table 9.** Retention of Total β-carotene in red palm-oil (RPO) based soups

| **Dish name** | **Raw Ingredients** | | | **Cooked dish** | | | |
|---|---|---|---|---|---|---|---|
| | Name | Amount (g) | β-carotene[1] (µg) | Amount (g) | Yield factor | β-carotene with RPO[2] (µg/100g) | β-carotene without RPO[1] (µg/100g) |
| *Stew* | Tatase | 69 | 1,587 | - | | - | 1,746 |
| | Chili pepper | 39 | 250 | - | | - | 244 |
| | Onion | 62 | - | - | | - | - |
| | Tomato | 183 | 1,142 | - | | - | 1,405 |
| | Palm oil | 200 | 137,360 | - | | 5,946 | - |
| | Seasoning | 4 | - | - | | - | - |
| | Salt | 2 | - | - | | - | - |
| | Water | 250 | - | - | | - | - |
| | Total | 809 | 140,761 | 370 | 0.46 | 5,946 | 3,395 |
| *Egusi soup* | Tatase | 108 | 2,484 | | | | 2,732 |
| | Chili pepper | 78 | 499 | | | | 488 |
| | Onion | 96 | - | | | | - |
| | Palm oil | 250 | 171,700 | | | 9,908 | |
| | Amaranthus | 200 | 5,780 | | | | 5,480 |
| | Melon | 60 | - | | | | - |
| | Salt | 10 | - | | | | - |
| | Water | 550 | - | | | | - |
| | Total | 1,352 | 180,463 | 935 | 0.69 | 9,908 | 8700 |

The carotenoid content of RPO (Red Palm Oil) are based on analysed value; [1] Values are based on the reported values in the West-African Food Composition Table [16]; [2] Values are based on laboratory analyses of cooked dish

**Table 10**. Effect of yellow cassava on vitamin A intake

| Variable | ß(exp) | 95% CI | p-value |
|---|---|---|---|
| Intervention group[1] | 1.57 | 1.43 – 1.72 | <0.0001 |
| Time point[2] | 1.06 | 0.97 – 1.16 | 0.21 |
| Group * time | 0.63 | 0.56 – 0.72 | <0.0001 |

[1]Yellow cassava group vs. white cassava group; [2]Time point 2 (after intervention) vs. time point 1 (during intervention).

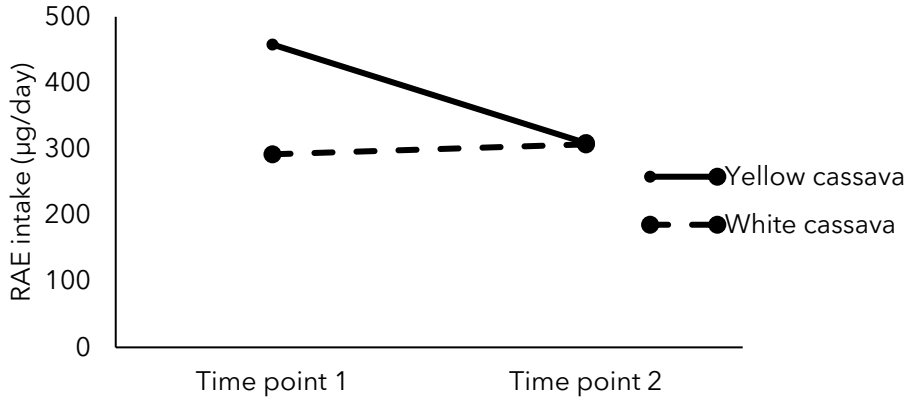


**Figure 8**. Estimated marginal mean RAE intake in the yellow cassava and white cassava groups. Time point 1= 1st round of dietary intake assessment (during intervention); Time point 2 = 2nd round of dietary intake assessment (after intervention).

Retention of β-carotene in palm oil-based soups are presented in **table 9.** Estimated β-carotene retention in stew and *egusi* vegetable soups were 6.8% and 9% respectively.

**Table 10** presents the effect of yellow cassava on vitamin A intake after adjusting for energy intake, age and gender. Both treatment group ($p<0.0001$) and time ($p<0.0001$) significantly explained differences in RAE intake, and their interaction terms showed statistical significance as well. The main effect of the yellow cassava group versus the white cassava group on RAE intake was Exp(β)= 1.57 (95% CI 1.43, 1.72), whereas the interaction effect of group and time showed that there was a 37% decrease in RAE intake in the yellow cassava group after the intervention. (Exp(β) = 0.63 (95% CI 0.56, 0.72). **Figure 8** shows the estimated marginal mean intake of RAE in the yellow cassava and white cassava groups during and after the intervention: during the intervention, the yellow cassava and white cassava groups had a mean intake of 458 µg and 292 µg RAE respectively, which declined to 309 µg in the yellow cassava group and increased to 307 µg in the white cassava group, after the intervention.

## DISCUSSION

This study aimed at estimating the contribution of pro-vitamin A biofortified (yellow) cassava to vitamin A intake in Nigerian pre-school children, both under experimental and free-living conditions. We found that vitamin A intake was 57% higher in the yellow cassava group compared to the control group during the RCT, whereas intake declined after the intervention. During the RCT, yellow cassava provided sufficient RAE for nearly all the children (96%). After the intervention, when children had returned to their habitual diets, replacement of white cassava with yellow cassava was estimated to still be able to provide for 32% of total vitamin A intake and to reduce inadequacy of intake to 0%.

The goal of pro-vitamin A biofortification programmes is to provide biofortified crops that will contribute at least 50% of the EAR for provitamin A in target populations [27]. Our present findings show that the TMS 07/0593 variety of yellow cassava with approximately 9µg/g (wet weight) β-carotene used in this study, met the set goal under experimental conditions as well as when simulated under free-living conditions. Since breeding programmes are continuously ongoing, newly released varieties can be expected to perform even better. Our findings are also consistent, although higher than another study by Talsma *et al* (2016), who, using a linear programming approach, showed that providing a school lunch with yellow cassava to children 6-12 years old could cover up to 47% of the Recommended Nutrient Intake (RNI) of the school children [10]. RNI are set as two standard deviations above the EAR, hence the lower value in their

study as compared to ours. Moreover, the methodological differences between the two studies could also be responsible for the differences in estimates.

An important contributor to vitamin A intake in the children's diets was red palm oil (RPO). Over 80% of the children consumed RPO at least once a day in one form or another. A different study around the same location showed that RPO consumption was ubiquitous and that about 43% of children consumed RPO six times per week or more [28]. Raw RPO can contain high amounts of β-carotene and has been reported to range between 37,300 to 100,060 µg/100g in West African diets [16]. To the best of our knowledge, the present study is the first to provide an estimate on β-carotene retention in commonly-consumed Nigerian soups cooked with RPO, although there are few studies that have reported the total carotenoid and β-carotene content of these dishes [29–32]. However, the average retention of β-carotene in RPO, estimated from this study may not be applicable to the entire Nigerian population, as cooking methods differ widely across ethnic groups in the country [33].

RPO was mostly used to prepare stews and vegetable soups, which were usually consumed along with a starch-based staple food. A comparison of previous studies on the carotenoid content of *egusi* vegetable soup showed that β-carotene varied between 204µg/100g [34] to 13,047µg/100g [30]. This large variation in carotenoid content can be partly explained by differences in cooking methods. For example, we observed that bleaching of RPO prior to cooking, was a common practice amongst mothers in the community where our study was conducted. It may therefore be erroneous to apply the β-carotene concentration values of similar soups, directly from food composition tables without accounting for cooking time and method, and consequently, without appropriate retention factors for RPO, which is the largest source of carotenoids in these dishes [31,35]. We estimated that RPO consumption contributed about 35% to total RAE intake, which was barely over half of the value reported by De Moura *et al* (2015), in a study on cassava intake among women and pre-school children in Akwa-Ibom, southern, Nigeria [35]. In that study, the contribution of palm oil to daily RAE was about three times higher than the estimated value in the present study. This disparity is probably because authors did not account for the loss of β-carotene from RPO upon heating since solid data for the β-carotene content of many Nigerian dishes are lacking. Based on the widespread consumption of RPO in Nigeria, further studies are needed to understand the amount of β-carotene that is retained from RPO-based foods under different cooking conditions. This will lead to more precise estimations of the contribution of RPO to vitamin A intake. Furthermore, it

would also be interesting to explore the possibility of adapting cooking habits such that β-carotene from RPO is better retained.

From our study, despite the contribution of RPO, approximately 43%of the children had inadequate vitamin A intake when consuming their habitual diets **(table 8)**. This clearly suggests that vitamin A intake is still lower than recommended in this population, a gap which can be filled by promoting biofortification and other food-based strategies to improve vitamin A intake.

For the conversion of β-carotene to retinol, we assumed a bioequivalence factor of 7:1 for biofortified cassava-based foods in the present study. Some studies have shown that the bioequivalence of pro-vitamin A from biofortified cassava can be as high as 4:1 based on experimental studies with single meals under highly controlled conditions in healthy adults (19). We used a more conservative value since the study was conducted in children living in a poor resource setting where malnutrition, intestinal parasite infestation and infectious disease are rife. If we would have used a higher conversion factor, the contribution of biofortified cassava to vitamin A intake would obviously have been even higher than our current estimates.



A very important strength of this study was that we measured nutrient intake during the intervention and afterwards, when the participants had returned to their usual diets. This provided a more accurate estimation of the contribution of yellow cassava to provitamin A intake during the intervention, as well as a realistic estimate of the potential increase in vitamin A intake that would occur if yellow cassava replaced white cassava. Other important strengths were the steps taken to improve the accuracy of nutrient intake estimation. First and foremost, we conducted laboratory analyses of biofortified food samples and commonly-consumed red palm oil-based foods, which provided more reliable nutrient composition data, especially for biofortified foods which were not available in the national food composition database. Secondly, we developed recipes for all biofortified foods and commonly-consumed local soups, because standard recipes were unavailable for most of the soups consumed in the community households. Thirdly, we estimated nutrient intake as a sum total of all individual ingredients used in the preparation of a particular dish. This method is deemed to be more reliable especially in rural communities in developing countries where standard recipes may not be available for many dishes [36]. Furthermore, we adjusted nutrient and energy intake distribution for day-to-day variation using repeated recalls. This provided a more accurate estimation of usual intake, by partially eliminating day-to-day variation.

This study also had some limitations. Firstly, to estimate the nutrient value of some foods, values from food composition tables from multiple countries were adopted because the Nigerian Food Composition table did not cover the composition of all foods consumed in the community. A food composition table in principle should be country-specific, thus using food composition tables from other countries may have also introduced some errors in nutrient intake estimation. In addition, information on fortification was unavailable in most of the food composition tables consulted. Therefore, the vitamin A content of some fortified flour-based snacks, sugars and oils where proper nutrition labels were absent, were not captured, thus contributing to errors. However, we expect that such errors would have been minimal because these food products were minimally consumed. For example, we were unable to compute possible vitamin A intake from possibly fortified flour used as an ingredient in biscuit, in which about 18% of the children consumed an average of 23 grams during the recall days. Moreover, compliance to mandatory food fortification for sugar and oil has been reported to be less than 20% in Nigeria [37]. For our simulation, we assumed that, after intervention, all white cassava in the children's diets would be replaced by biofortified varieties. However this may not be the case, as some people may still have a high preference for white cassava. Finally, we did not account for seasonal variation in vitamin A intake. For example, certain pro-vitamin A-rich foods, such as mango, was unavailable during the season the study was conducted. This may also have contributed to errors in estimating the habitual intake of vitamin A as well as the percentage of children below the EAR.

In conclusion, yellow cassava contributed ~40% of the total intake of vitamin A and has the potential to reduce the percentage of children at risk of inadequate intakes of vitamin A to a low level. Yellow cassava is therefore recommended as a good source of provitamin A in cassava-consuming regions.

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# Chapter Five

## Comparison of serum vitamin A adjustment methods in Nigerian pre-school children at risk of malaria infection

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## ABSTRACT

**Background:** The assessment of vitamin A status is still a challenge in populations with high malaria and other infections, due to parasite-induced inflammation, which temporarily reduces serum retinol binding protein concentration. Several correction methods have thus been developed to adjust for this transient reduction.

**Objective:** The study compared results obtained from using three RBP correction methods to calculate adjusted RBP. Secondly, we assessed the effect of malaria and inflammation on RBP concentration among pre-school children from South-west Nigeria.

**Methods:** The study was a cross-sectional study with 427 children under-five years old, who participated in the screening phase of a randomized-controlled trial on the efficacy of pro-vitamin A biofortified cassava. Serum RBP, C-reactive protein and α-1-glycoprotein (AGP) were analysed using a sandwich ELISA technique, malaria parasitaemia was measured using a real-time quantitative PCR. Adjusted RBP concentrations were calculated by various methods that are commonly used for the adjustment of serum retinol concentration, here referred to as the Thurnham, the BRINDA and the Palmer correction methods. To compare the relationship between malaria and other forms of infection, participants were categorized into no parasitaemia, low/moderate (1-999 parasites/μL whole blood) and high parasitaemia (>1000 parasites/μL whole blood).

**Result:** Participants had a mean CRP concentration of 10.5 mg/L ± 17.3 mg/L (44% >5 mg/L), and mean AGP concentration of 1.2 g/L ± 0.6 g/L (56% > 1g/L). The Thurnham and Palmer adjustment methods gave similar estimates of adjusted RBP (0.95 μmol/L ± 0.28 μmol/L and 0.96 μmol/L ± 0.28 μmol/L, respectively), while the BRINDA method gave a higher estimate of 1.03 ± 0.3 μmol/L ($p<0.0001$). Thirty-seven percent of participants (n=158) had no parasitaemia, 56% (n=239) had moderate malaria parasitaemia while 13% (n=30) had high malaria parasitaemia. CRP concentrations in the no-parasitaemia group was 5.1 mg/L ± 10.8; low/moderate parasitaemia, 11.6 mg/L ± 16.8 and high parasitaemia, 27.4 ± 26.0 mg/L ($p<0.0001$). Children with no inflammation and no parasitaemia (n=100) had a higher mean RBP concentration of 0.98 ± 0.27 μmol/L ($p=0.04$) compared with children with low/moderate parasitaemia (0.89 ± 0.24 μmol/L; n=51).

**Conclusion:** Thurnham and Palmer adjustment methods gave similar estimates of adjusted RBP compared to the BRINDA method. The presence of malaria parasitaemia appears to contribute to the reduction of serum RBP independently of inflammation status.

## Introduction

Inflammation status is a major determinant of blood retinol and retinol binding protein (RBP) concentrations [1,2]. RBP in the bloodstream is responsible for the transport of retinol from the liver to extra-hepatic tissues [3] and is commonly used as a proxy biomarker of vitamin A status [4]. Inflammation refers to the physiological process by which the body fights against pathogenic infections [5]. During inflammation, due to acute phase reactions, there is an increased production of inflammatory proteins such as c-reactive protein (CRP) and α1-glycoprotein (AGP), and a transient reduction in plasma RBP and retinol concentrations [6,7]. This concept has been described as inflammation-induced hyporetinolemia [8,9]. Similarly, malaria infection leads to an increased production of CRP in the plasma, [10] which in principle may lower plasma RBP and retinol concentrations. Although the effect of inflammation on RBP is quite clear, it is less clear if the presence of malaria and other infections have a combined effect on plasma RBP and/or retinol concentrations.

The assessment of vitamin A status based on plasma RBP and retinol concentrations is cumbersome in populations endemic with malaria and other pathogenic infections [2], and may lead to an over estimation of the true prevalence of vitamin A deficiency unless values are corrected for the presence of inflammation and infection. Recently, mathematical methods have been developed to make such corrections in order to estimate vitamin A status more accurately. Although these methods employ different principles, their common underlying principle is to use the plasma RBP or retinol concentration of an uninfected sample of a population to correct or adjust the values of the infected sample of the same population [1,11–13]. Some of these methods only consider markers of inflammation in their adjustment method [1,11,13,14] whereas others report an independent effect of malaria infection on vitamin A status indicators [15,16] The mechanism by which malaria infection would lead to reduced plasma retinol and/or RBP concentrations apart from the inflammatory process is not very clear. Disease-specific differences in the synthesis, turnover and distribution of RBP have been suggested by some [16], while others have suggested that retinol may be specifically utilized during malaria for an increased phagocytosis of parasitized erythrocytes [17]. This has implications for whether or not to independently account for malaria when adjusting blood concentrations of RBP or retinol [15]. Some studies have shown that the effect of malaria status on blood concentrations of retinol is independent of inflammation [2,18,19], thus providing a rationale for an additional correction when malaria is present.

To provide further evidence for this, we here examined the relationship between malaria parasitaemia, inflammatory indicators and plasma RBP

concentrations in a malaria and inflammation endemic population . For this, we compared three mathematical adjustment methods, here referred to as the Thurnham method [20], the BRINDA methods 1 and 2; [19], and the Palmer method [13].

## Methods

### Study Population

The current analyses are based on data collected during the screening phase of a randomized controlled trial (RCT) designed to study the efficacy of biofortified cassava among pre-school children aged 3 – 5 years in three rural communities in south-western Nigeria. The study is registered at www.clinicaltrial.gov with the registration code NCT02627222 The detailed descriptions of the study population and methods have been described in chapter two. In brief, eligible children were invited to participate in the study during a community mobilization programme. A total of 439 children were screened, based on the eligibility criteria described in chapter two. Children's body temperature were taken during screening with a digital forehead thermometer (Omron Healthcare Co., Ltd, Netherlands). All screened participants were enrolled by either a parent or guardian who provided both verbal and written consent. A total of 427 blood samples were collected and analysed for RBP, C-reactive protein, α-1-glycoprotein and malaria parasites.

### Biochemical assessments and laboratory analyses

Whole blood samples were collected by venepuncture into heparinized vacutainers (Becton and Dickinson, Carlifornia, USA) by phlebotomists from the University College Hospital, Ibadan, Nigeria. Vacutainers were quickly stored in dark containers on ice and were transferred immediately to the field laboratory for centrifugation. Plasma was aliquoted into microvials and stored in liquid nitrogen at -196°C. Samples were later shipped on dry ice to the VitMin laboratory (Willstaett, Germany), where they were analysed for concentrations of RBP, CRP and AGP, using the combined sandwich ELISA technique described by Erhardt *et al.,* (2004) [21]. RBP values were standardised for retinol concentration based on control samples with a known RBP: retinol ratio, which were run along with the other samples. In the present study, RBP concentration was also compared with HPLC retinol concentration, measured during the baseline phase of the RCT in approximately 40% of the participants.

Malaria parasitemic load was measured through the detection and extraction of *P.falciparum* -specific DNA from erythrocytes in whole blood using a real time quantitative polymerase chain reaction (RTQ-PCR) technique. The full details of the procedures have been described by Hermsen *et al*, (2001) [22].

**Definitions and calculations**

Inflammation was defined as blood CRP > 5mg/L and/or AGP> 1g/L; no inflammation, CRP ≤ 5 mg/L and/or AGP < 1 g/L. [20]. For malaria infection, no parasitaemia was defined as children with malaria parasite count of zero; low/moderate parasitaemia, as malaria parasitaemia count > 0 and <1,000 parasites/µl whole blood; high parasitaemia, as malaria parasitaemia >1,000 parasites/µl whole blood. These categories reflect the severity of malaria infection, and were derived from the WHO plus system of counting parasitaemia. [23].VAD was defined as plasma RBP < 0.7µmol/L, with unadjusted RBP concentration standardised for retinol (in µmol/L), as reported by the VitMin laboratory; and adjusted RBP as inflammation and/or malaria-adjusted plasma RBP concentration in µmol/L.

**Statistical Analysis**

Data was analysed using STATA 15 software (Stata Corp, College Station TX). All variables used in the analyses were tested for normality using a Q-Q plot and/ or visual inspection. Non-normal variables (RBP, CRP, AGP) were log-transformed using their natural logarithms. For all inferential statistical analyses, log-transformed variables were back-transformed by taking their exponentials. Means calculated from back-transformed variables were reported as geometric means. One-way analysis of variance (ANOVA) was used to test for statistical differences among the adjusted mean RBP values derived from the Thurnham, BRINDA and Palmer adjustment methods. This was followed by a pairwise comparison of means to identify group means that significantly differed from each other. To estimate the effect of malaria on unadjusted RBP, a univariate regression model was constructed, using the natural log of the unadjusted RBP concentration (lnRBP) as the outcome variable, and the binary value of malaria parasite infection (i.e. malaria present or absent)as the predictor variable. The effect of malaria was thus expressed as the exponential beta of the coefficient of the predictor variable and presented as the percentage change. Independent t-test analysis was used to test for difference in plasma RBP concentration between participants in the no-parasitaemia and low/moderate parasitaemia group who had no inflammation Levene's test of equality of variance was conducted to confirm variance homogeneity between no-parasitaemia and low-moderate parasitaemia groups.

**Thurnham Adjustment**

For the Thurnham adjustment [20], CRP and AGP inflammation biomarkers were used to calculate adjusted RBP. Four adjustment groups were generated to represent different stages of inflammation as follows: reference category or no inflammation group (CRP ≤ 5mg/L, AGP ≤ 1g/L), incubation group (CRP > 5mg/L, AGP ≤ 1g/L), early convalescence group (CRP > 5mg/L, AGP > 1g/L), and late convalescence group (CRP ≤ 5mg/L,

AGP > 1 g/L). To calculate the adjustment factor for a group, the geometric mean of RBP for that group was divided by the geometric mean RBP of the reference category.

**BRINDA Adjustment**

A continuous adjustment using the regression correction was used as the choice correction method as opposed to using fixed correction factors, because it accounted for the entire spectrum of inflammation [19]. Using this approach, two different models were built to calculate adjusted RBP as follows:

Model 1: RBP(adj)= RBP(unadj) - β1(CRP[obs] - CRP[ref]) - β2(AGP[obs] - AGP[ref])
Model 2 :RBP(adj)= RBP(unadj) - β1(CRP[obs] - CRP[ref]) - β2(AGP[obs] - AGP[ref]) - β3 malaria

Where RBP(adj)=adjusted RBP value; RBP(unadj)=unadjusted RBP value; β1=CRP regression coefficient; CRP(obs) = observed CRP (from dataset); CRP(ref)=reference CRP i.e. the maximum value of the lowest CRP decile; β2=AGP regression coefficient; AGP(obs)=observed AGP (from dataset); AGP(ref)=reference AGP (i.e. the maximum value of the lowest AGP decile; β3=malaria regression coefficient; malaria=dichotomous value of malaria (i.e. present or absent based on RTQ-PCR analysis). β1 and β2 values were generated by constructing a linear regression model where RBP was the outcome variable and CRP and AGP were predictor variables. Model 1, was used to calculate inflammation-adjusted RBP without accounting for malaria, while model 2 was used to calculate both inflammation and malaria-adjusted RBP.

**Palmer Adjustment**

To calculate adjusted RBP, using the Palmer adjustment, we used the CRP-only model as described by Palmer *et al,* (2008), because this model explained the highest percentage of the variance in retinol in both malaria and non-malaria seasons in their study [13]. In this model, three categories of CRP were defined as follows: reference (CRP<5mg/mL), moderate inflammation (CRP 5-15mg/mL), and high inflammation (CRP>15mg/L). Adjustment factors were calculated as a ratio by dividing the geometric mean RBP concentration of the reference category by the geometric mean RBP concentration of the moderate inflammation category and high inflammation categories, respectively.

**RESULTS**

The demographic and biochemical characteristics of the study participants are presented in **Table 11.** Forty-seven percent (n=199) of participants were girls with a mean age of 45 months, while 53% percent (n=228) were

boys, with a mean age of 46 months. The average body temperature was 36.4 degree Celsius and only 6 participants had body temperatures above 37.5 degree Celsius. Participants had a mean CRP and AGP concentration of 10.5 mg/L ± 17.3 mg/L and 1.2 g/L ± 0.6 g/L, respectively. Forty-four percent of participants (n=188) had plasma CRP > 5 mg/L, while 57% (n=242) had plasma AGP >1 g/L. Thirty-seven percent of participants (n=158) had no parasitaemia, 56% (n=239) had moderate malaria parasitaemia while 13% (n=30) had high malaria parasitaemia. A disaggregation of inflammation markers by malaria parasitaemia group showed a mean CRP concentration of 5.1 mg/L ± 10.8 mg/L in the no-parasitaemia group, 11.6 mg/L ± 16.8 mg/L in the low/moderate parasitaemia group and 27.4 mg/L ± 26.0 mg/L in the high parasitaemia group (p<0.0001). (**Table 11)**.

**Table 11:** Demographic and biochemical characteristics of participants

| Characteristics | [1]No Parasi-taemia | [2]Low/ moderate Parasitaemia | [3]High Parasi-taemia | Total |
|---|---|---|---|---|
| Participants, n (%) | 158 (37) | 239 (56) | 30 (13) | 427 |
| Male, n (%) | 79 (50) | 127 (53) | 22 (73) | 228 (53) |
| Age in months ± SD | 44 ± 10 | 47 ± 12 | 47 ± 12 | 46 ± 11 |
| CRP, (mg/L) | 5.1 ± 10.8 | 11.6 ± 16.8 | 27.4 ± 26.0 | 10.5 ± 17.3 |
| AGP, g/L | 1.0 ± 0.7 | 1.6 ± 0.8 | 2.1 ± 1.0 | 1.2 ± 0.6 |
| Elevated CRP > 5mg/L, n (%) | 34 (22) | 129 (54) | 25 (83) | 188 (44) |
| Elevated AGP > 1g/L, n (%) | 51 (32) | 164 (68) | 26 (86) | 242 (57) |

[1]RTQ-PCR parasite count=0; [2]RTQ-PCR parasite count > 0 and < 1,000/µl whole blood; [3]RTQ-PCR parasite count > 1000/µl whole blood.

**Table 12** shows the mean RBP concentration during different stages of inflammation and malaria infection. Children with no inflammation and no parasitaemia (n=100) had a higher mean RBP concentration of 0.98 ± 0.27 µmol/L (p=0.04) compared with children with low/moderate parasitaemia (0.89 ± 0.24 µmol/L; n=51. There was a strong correlation between RBP and plasma retinol concentration measured at baseline during the RCT (r = 0.92; p<0.0001).

Based on the Thurnham and Palmer method, overall average of adjusted RBP concentrations were 0.95 µmol/L ± 0.28 µmol/L and 0.96 µmol/L ± 0.28

μmol/L, respectively. Both the Thurnham and Palmer estimations were higher than the unadjusted RBP values **(table 13)**. The BRINDA adjustment methods model 1 & 2 resulted in a higher adjusted RBP concentration of 1.03 ± 0.3 μmol/L and 1.00 ± 0.29 μmol/L, respectively **(table 13)**. When comparing the Thurnham adjustment method with the BRINDA models, it showed a statistically significant difference only with model 2 ($p<0.001$), where malaria was adjusted for **(table 14)**. Similarly, comparing the Palmer method with the BRINDA methods, showed a statistically significant difference only in model 2 ($p<0.001$) where malaria was adjusted for. Additionally, the estimation of VAD based on the Palmer and Thurnham adjustment method did not differ from each other with an estimated VAD of 16% and 17% ($p=0.87$), respectively, whereas the BRINDA adjustment method gave an estimated prevalence of VAD of 10% and 12% for models 1 & 2 respectively **(table 13)**. In an unadjusted univariate analysis **(table 13)**, malaria parasitaemia predicted a decrease of 13% in RBP concentration ($p<0.001$), while this decrease was 6% after adjusting RBP values according to the Thurnham method ($p=0.042$); 5% according to the Palmer method ($p=0.09$); 4% according to BRINDA model 1 ($p=0.14$); and 2% according to BRINDA model 2 ($p=0.47$).

**Table 12:** Unadjusted RBP concentrations at different stages of inflammation and malaria infection

| Category | n | No Parasitaemia | n | Low/moderate Parasitaemia | n | High Parasitaemia | n | Total |
|---|---|---|---|---|---|---|---|---|
| No inflammation: CRP ≤ 5 mg/L; AGP < 1 g/L | 100 | 0.98 (0.92, 1.03)[a] | 51 | 0.89 (0.82, 0.95)[b] | 1 | 0.86 | 152 | 0.95 (0.91, 0.99) |
| Incubation stage: CRP > 5 mg/L; AGP < 1 g/L | 7 | 0.74 (0.61, 0.86) | 20 | 0.67 (0.59, 0.75) | 3 | 0.94 (0.37, 1.52) | 30 | 0.72 (0.65, 0.78) |
| Early convalescence: CRP > 5 mg/L; AGP > 1 g/L | 27 | 0.79 (0.68, 0.90)[a] | 108 | 0.78 (0.74, 0.82)[b] | 22 | 0.69 (0.60, 0.78) | 157 | 0.77 (0.73, 0.80) |
| Late convalescence: CRP ≤ 5mg/L; AGP > 1 g/L | 24 | 1.03 (0.91, 1.14) | 55 | 0.96 (0.87, 1.05) | 4 | 0.86 (0.40, 1.32) | 83 | 0.98 (0.91, 1.05) |

[a,b] Groups with different letters indicate statistically significant differences (i.e. $p<0.05$);

**Table 13: Comparison of RBP adjustment methods and effect of malaria infection on adjusted and unadjusted RBP**

| | Unadjusted | Thurnham adjustment | BRINDA adjustment[1] | | Palmer adjustment |
|---|---|---|---|---|---|
| | | | Model 1 | Model 2 | |
| RBP (µmol/L)[2] | 0.87 ± 0.27[a] | 0.95 ± 0.28[b] | 1.00 ± 0.29[c] | 1.03 ± 0.3[c] | 0.96 ± 0.28[b] |
| Vitamin A Deficiency, n (%)[3] | 123 (29) | 71 (17) | 43 (10) | 50 (12) | 66 (16) |
| Change in RBP due to malaria, % (95% CI) | -13 (-18, -7) | -6 (-11, 0) | -4 (-9, 1) | -2 (-7, 4) | -5 (-10, 0) |

[1]Model 1 is BRINDA correction method unadjusted for malaria; Model 2 is BRINDA correction method adjusted for malaria

[2]Represents the arithmetic mean of RBP concentration

[3]Plasma RBP < 0.7µmol/L

Groups with different letters indicate statistically significant differences (P<0.05).

**Table 14: Mean RBP comparison among different adjustment methods**

| Adjustment methods | Mean RBP difference ratio | Confidence interval | P-value |
|---|---|---|---|
| Thurnham vs. unadjusted | 1.23 | 0.71 – 0.92 | <0.0001 |
| Palmer vs. unadjusted | 1.27 | 0.69 – 0.89 | <0.0001 |
| BRINDA (model 1) vs. unadjusted | 1.41[a] | 1.24 – 1.60 | <0.001 |
| BRINDA (model 2) vs. unadjusted | 1.52[a] | 1.34 – 1.72 | <0.0001 |
| Thurnham vs. Palmer | 0.97 | 0.87 – 1.09 | 0.939 |
| BRINDA (model 1) vs. Thurham | 1.14 | 1.01 – 1.28 | 0.05 |
| BRINDA (model 2) vs. Thurnham | 1.22 | 1.08 – 1.38 | <0.0001 |
| BRINDA (model 1) vs. Palmer | 1.11 | 0.98 – 1.25 | 0.184 |
| BRINDA (model 2) vs. Palmer | 1.19 | 1.05 – 1.35 | 0.001 |

Mean difference between models is expressed as ratio

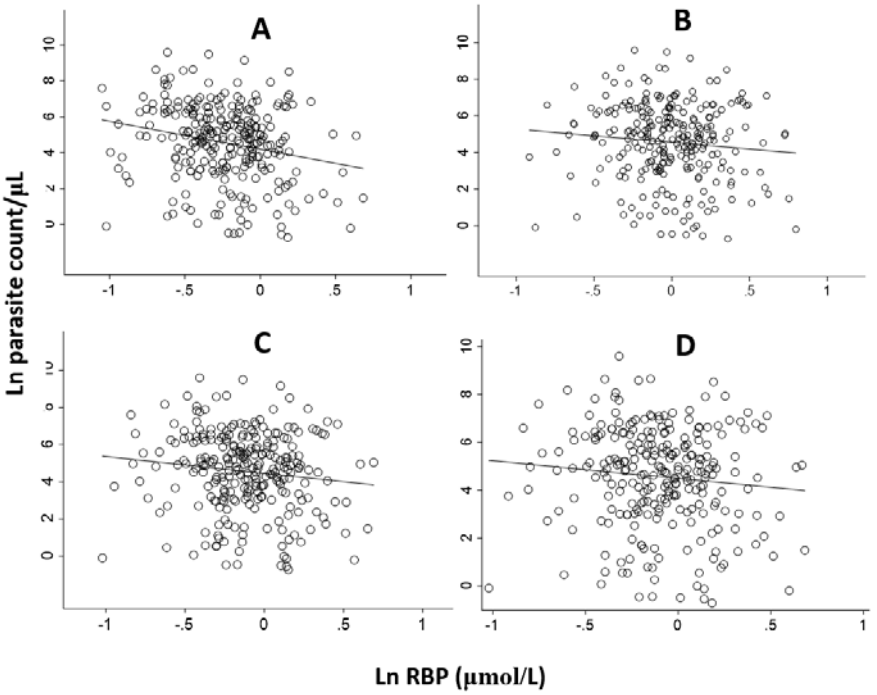


**Figure 9**: Relationship between malaria parasite infection and RBP in 4 different adjustment models. A= Unadjusted RBP; B= BRINDA adjustment: C=Thurnham adjustment; D=Palmer adjustment

## DISCUSSION

There has been a rising global research effort to reduce the potential bias introduced by inflammation during the quantitation of serum retinol concentrations and the estimation of vitamin A status. This attempt has given rise to several adjustment methods, however there is presently no consensus on the optimal model that should be used for retinol or RBP adjustment [13]. A comparison of available adjustment methods is thus an important step towards agreeing on a consensus. In the present study, we applied three different adjustment methods described by Thurnham *et* al, (2015) [20], Larson *et al.*, (2017) (BRINDA) [19], and Palmer *et al*., (2018) [13] to plasma RBP collected during the screening phase of a randomized control trial. A comparison of all three models showed greater similarity between the adjusted RBP estimated using the Thurnham and Palmer models, compared to the BRINDA model 1. Similarly, based on the estimation of VAD, prevalence was closer in the Palmer and Thurnham adjustment compared to the BRINDA adjustment. A possible reason for the disparity observed in the BRINDA-adjusted RBP concentration could be in the nature of the regression model used. The BRINDA adjustment model

assumes linearity between retinol and CRP/AGP, which may not always be the case. There is thus an inherent potential to over-adjust by applying the same regression coefficient to every individual [13]. In this study, following the BRINDA methodology, we applied adjustment factors to 83% of participants who were at or above the first decile of the log-transformed AGP and CRP concentrations. This may have resulted in the higher estimations of adjusted RBP in this group.

A few studies have suggested that the presence of malaria infection may lead to a reduction in retinol or RBP, independently of CRP or AGP-induced inflammation status [13,18]. In agreement with these studies, our current findings suggest a similar trend. In addition, a comparison of RBP concentrations among children without inflammation in the no-parasitaemia and low/moderate parasitaemia group (i.e. CRP≤5mg/L; AGP<1g/L) showed a significant reduction in RBP However, separating the inflammatory effects of malaria from other infections may be quite difficult in practice because both malaria and other forms of infection lead to an increase in plasma CRP concentrations. Moreover, one cannot completely rule out all forms of infection solely based on a CRP cut-off of 5mg/L. A comparison of all the adjustment methods suggest that after adjustment, the effect of malaria on RBP was still significant (p=0.042) in the Thurnham adjustment. However, malaria was not significantly associated with RBP concentrations adjusted using the BRINDA (p=0.5) and Palmer (p=0.09) methods.

We observed different fluctuations in RBP concentrations under established inflammatory conditions. For example, following the four stages of inflammation described by Thurnham *et al*, [20] during acute phase responses, in the incubation stage of infection, when CRP concentration was high, RBP concentrations were lowest in both the malaria and the non-malaria group, with no statistically significant difference between the two groups This observation is consistent with previous studies [14,24] that have reported that the concentration of positive acute phase proteins such as CRP increases during malaria infection as well as other forms of infection. However, we observed a marked difference during the late convalescence category (i.e. CRP≤5mg/L; AGP>1g/L; when AGP concentration is still high, although CRP concentrations have become low. During this stage, unadjusted RBP concentrations are highest in the no-parasitaemia and low/moderate parasitaemia groups, with the RBP concentrations being higher than in the reference group (i.e. CRP<5mg/L; AGP<1g/L). Although unexpected, a similar trend was reported by Wessels *et al* (2014) and Palmer *et al*, (2018) and this further questions the need to statistically adjust for RBP or retinol concentration during the late convalescence stage of

infection, when using the Thurnham correction method for RBP or retinol. During inflammation, the blood concentration of positive acute phase proteins such as CRP and AGP are expected to increase, whereas negative acute phase proteins such as RBP are expected to reduce [25]. It is therefore not clear if our observation of an increased RBP during the late convalescence stage of infection as classified by Thurnham *et al,* has any biological implication for malaria infection.

An important limitation of the study was limited sample size of some combinations of categories of parasitaemia concentration and inflammation It is also likely that the inconsistency in RBP concentrations in this group was due to the small group sample sizes, which is also reflected in the large confidence intervals. Secondly, we observed a malaria transmission prevalence of 63% during this study, which compares to that of Palmer *et al* (2018) high transmission season [13]. However, parasite densities were still relatively low in our study because children were afebrile and asymptomatic for malaria.

A major strength of the study was the strong correlation between the RBP concentration used in the study and serum retinol concentrations measured at baseline during the randomized controlled trial. This correlation implies that our results can be extrapolated to plasma/serum retinol concentrations. Furthermore, the use of real-time quantitative PCR to quantify malaria parasites provided a more objective basis for quantitation.

Thurnham and Palmer adjustment methods gave similar estimates of adjusted RBP compared to the BRINDA method. The presence of malaria parasitaemia appears to contribute to the reduction of serum RBP independently of inflammation status.

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# Chapter Six

General Discussion

The overall aim of this thesis is to provide proof of principle of the efficacy of biofortified (yellow cassava) to increase serum retinol concentration. The main findings of the thesis are summarized in this chapter, followed by a critique of the methodologies employed. Finally, the public health implications of our findings in low and middle-income populations are discussed, and suggestions are provided for future research.

The main findings of the study are presented in table 1. In summary, we found that daily consumption of yellow cassava modestly increased serum retinol concentration, as well as serum haemoglobin concentrations in Nigerian preschool children. However, no effect was found on serum β-carotene concentrations. We did not detect any difference in gut permeability between experimental and control groups **(chapter 2)**, however, it was observed that sugar permeability ratios – the indicator used to measure gut permeability - varied with the volume of urine voided **(chapter 3)**. We compared the dietary vitamin A intake during and after the intervention in the yellow and white cassava groups **(chapter 4)** and determined the potential contribution of yellow cassava to total vitamin A intake. We found that if white cassava were replaced by yellow cassava, the potential contribution of yellow cassava to total vitamin A intake would be about 32%, which covered up to 63% of the EAR for children 3 – 5 years. Finally, we found that malaria significantly contributed to a decrease in RBP concentration among preschool children, and combined with other forms of inflammation was an important effect modifier of serum retinol concentration. As a result, adjustment methods are always needed in studies with serum retinol or retinol binding protein as the primary outcome. A comparison of three common serum retinol adjustment methods **(chapter 5)**, thus showed that the Thurnham and Palmer adjustment methods gave similar estimates of RBP, compared with the BRINDA adjustment method, which gave a higher estimate.

**A critique of applied methodology**

An initial critique of the strengths and weaknesses of the methodologies applied in this thesis has been discussed in the individual chapters. In this section, we discuss the various methodologies relevant to the internal and external validity of the various studies. By examining the internal validity of the individual studies in the thesis, we are interested in discovering if our findings are attributable to the independent variables identified, and not to some other causes that were inadvertently not measured.

**Possible threats to internal validity and how they were addressed in the studies.**

**A. Selection bias**

**(i) Selection Bias in the randomized controlled trial**

Selection biases are threats to the internal validity of a study which come from any error in selecting the study participants and/or from factors affecting study participation[1]. Selection bias may occur when subconsciously or otherwise, an investigator's knowledge of the treatment code influences treatment allocation [2–4]. In the efficacy trial **(chapter 2)**, selection bias could have occurred during allocation of participants to groups. However, this possibility was addressed through a blind randomization using an allocation code based on a list of random numbers generated by someone not involved in the field work [4]. Selection bias could also occur when the study population does not represent the target population. In the context of the efficacy trial, an example of a target population is a population of pre-school children deficient in vitamin A. In this context, one could argue that since the prevalence of VAD was just 10% in the study population, there may have been a selection bias, whereby recruited participants were not vitamin A deficient. However, we did not expect that this would have affected the result in any way, because randomization was properly conducted, and non-vitamin A deficient participants were randomly distributed in both control and experimental groups.

**Table 15: Main findings**

| Thesis Chapter | Study design, population & outcome | Objectives | Main result |
|---|---|---|---|
| Chapter 2 | Randomized Controlled Trial<br>Population: Preschool children, 3-5 years, attending a study-specific preschool.<br>Study outcomes: serum retinol, serum β-carotene, haemoglobin, lactulose mannitol ratio. | i. To determine the efficacy of yellow cassava consumption on serum retinol concentration.<br>ii. To determine the effect of yellow cassava consumption on serum β-carotene concentrations.<br>iii. To determine the effect of yellow cassava consumption on haemoglobin concentration.<br>iv. To determine the effect of yellow cassava consumption on gut permeability. | i. Moderate effect. Mean difference in serum retinol between groups: (0.06 µmol/L (95% CI: 0.004 – 0.124).<br>ii. No effect. Mean difference in β-carotene concentrations between groups: 3.9% (95% CI: -0.6%, 8%).<br>iii. Significant effect. Mean difference in haemoglobin concentration between groups: 3.08g/L (95% CI: 0.38 – 5.78.).<br>iv. No effect. Lactulose mannitol ratio between groups: 0.002 (95% CI: -0.089 – 0.092). |
| Chapter 3 | Cross-sectional study;<br>Population: preschool children, 3-5 years, attending a preschool designed for the study.<br>Study outcome: intestinal permeability ratio (LMR, LRR, SMR and SRR) | i. To determine the effect of voided urinary volume on intestinal permeability ratios in apparently healthy children. | i. Indicators of intestinal permeability in children varied with the volume of urine voided.<br>ii. Lactulose-mannitol ratio ($p<0.001$), lactulose-rhamnose ratio ($p<0.001$) and sucrose-rhamnose ratio ($p<0.002$) were all significantly higher in children who voided lower volumes of urine compared with children who voided larger volumes of urine.<br>iii. Urinary volume and voiding status combined, explained 13%, 23% and 7% of the variation observed in lactulose-mannitol, lactulose-rhamnose and sucrose-rhamnose ratios respectively. |

| | | | |
|---|---|---|---|
| Chapter 4 | Randomized Controlled Trial and cross-sectional study.<br>Population: preschool children, 3-5 years, attending a preschool designed for the study. | i. To determine median RAE intake between yellow cassava and white cassava groups during and after intervention. | i. During intervention, median RAE intake was 536µg/day in the YC group and 301µg/day in the WC group ($p<0.0001$). The interaction effect of group and time showed a 37% decrease in RAE intake in the YC group after the intervention (Exp β=0.63 [95% CI 0.56, 0.72]). |
| | Cross-sectional study | ii. To determine the potential contribution of yellow cassava to total dietary retinol activity equivalent (RAE) intake if white cassava is replaced by yellow cassava among pre-school Nigerian children. | ii. If WC was replaced by YC after the intervention, the potential contribution of yellow cassava to total RAE intake was estimated to be ~32%. |
| Chapter 5 | Cross-sectional study.<br>Population: preschool children, 3-5 years in a malaria-endemic population.<br>Study outcome: retinol binding protein | i. To compare three vitamin A adjustment methods for inflammation and malaria in Nigerian pre-school children at risk of malaria infection. | i. The Thurnham and Palmer adjustment methods gave similar estimates of adjusted RBP (0.95 ± 0.28 µmol/L and 0.96 ± 0.28 µmol/L respectively), while the BRINDA method gave a higher estimate of 1.03 ± 0.3 µmol/L ($p<0.0001$). |
| | | ii. To determine the effect of malaria and inflammation on retinol binding protein concentration among preschool children in a malaria-endemic population. | ii. Children with low/moderate parasitaemia without infection had a lower mean RBP concentration (0.89 ± 0.24 µmol/L), compared with children with no inflammation and no parasitaemia (0.98 ± 0.27 µmol/L; $p=0.04$). |

LMR= Lactulose-mannitol ratio; LRR=Lactulose-rhamnose ratio; SRR=Sucrose rhamnose ratio

A third way in which selection bias may have occurred was through the exclusion of participants. During the entire study, out of 568 participants screened, 250 (44%) were excluded either because they were too old or too young, and 159 children were excluded because they did not show up, did not give consent, were no longer interested, had low attendance/consumption, or left study area. Before randomization, most of the drop outs were due to participants not meeting the study inclusion criteria, therefore the chances of selection bias at this stage were considered very low. After randomization, only 17(~10%) of the participants were lost, in addition to 3 blood sample losses. These were almost equally distributed in the control and experimental groups, with 8 in the yellow cassava group and 9 in the white casava group. Overall, we conclude that the total number of non-responses was very small and would not have significantly altered the result obtained in the efficacy trial.

**(ii) Selection bias in the longitudinal and cross-sectional studies**

In the longitudinal study on the contribution of biofortified cassava to vitamin A intake **(chapter 4)**, only randomized participants were included in the study. Assuming we had included all the children in the community, would our results have been the same? Perhaps not. There may have been an increase or decrease in RAE intake of other participants not included in the study, which would have been a source of bias. However, we do not think this would have changed the result of the study significantly. Because the community members were generally homogenous with regards to important socio-economic indicators.

In the cross-sectional studies **(chapters 3 & 5)**, the possible effect of non-response bias on the result obtained, is also unlikely because the studies were methodological studies of biological mechanisms, which are less prone to selection bias. However this does not completely rule out bias because participants were not randomized.

**B. Information bias**

In the randomized controlled trial in chapter two, information bias could have occurred because of non-blinding of participants [5]. Due to the nature of the intervention, a double-blind control of both participants and the research team was impossible because of the bright yellow colour of the biofortified cassava. There is a possibility that based on the knowledge provided by the research team to parents about the health benefits of biofortified foods, they encouraged their children to consume more biofortified foods in the pre-school or provided them with more vitamin A foods at home If children consumed more vitamin A foods at home, this would have introduced a source of bias. However, judging from the observation that after the intervention, when the participants discontinued the consumption of the biofortified cassava, there was no difference in the

total RAE consumed, the bias resulting from such information must have been negligible. Furthermore, during analyses, all sample were blinded to the analyst, thus eliminating the possibility of information bias during sample analyses.

### C. Measurement bias

Measurement biases are those biases associated with the measuring instruments/tools used during the study, which may be a potential threat to the internal validity of the study [6].

#### (i) Measurement bias in the randomized controlled trial

In **(chapter 2)**, the initial study outcome had to be changed from total body retinol pool to serum retinol. Serum retinol is homeostatically controlled by liver enzymes [7–9], and only correlates with hepatic vitamin A stores in the deficient range ( below 0.7 µmol/L) and in the excessive range (above 2 µmol/L) [10]. Little is known about the linearity between hepatic vitamin A stores and serum concentration at intermediary concentrations. At endline, the mean adjusted serum retinol concentration was 0.96 µmol/L. At this concentration, it is possible that the effect of homeostasis was already present. On the one hand, the effect of retinol homeostasis may have biased our estimates of vitamin A status and deficiency. However, previous efficacy trials on provitamin A fortification have shown that a negligible effect of provitamin A biofortification on serum retinol concentrations, shows greater effect in hepatic vitamin A stores [11]. It is therefore more likely that we underestimated the real effect on vitamin A status in chapter two.

#### (ii) Measurement bias in the observational studies

In the assessment of small intestine permeability using sugar recovery tests (chapter three), it was observed that the estimation of gut permeability varied with urinary volume. There was a possibility that this additional source of variation could bias our estimation of sugar permeability ratios between the intervention groups. For example, for every 10% increase in urinary volume, lactulose mannitol ratio reduced by about 3.3% and lactulose rhamnose ratio reduced by about 4.5%. Although we adjusted for this statistically in **chapter 2**, we still found no difference in gut permeability between experimental and control groups in the RCT reported in **chapter 2**

The dietary assessment study reported in **chapter 4** presents some classical threat to internal validity, including recall bias and possible under-reporting or over reporting of food intake associated with the 24-hr dietary recall method [12–14]. Furthermore, errors associated with food composition tables may also have been introduced [15]. Several precautions were taken to reduce bias, including multiple trainings of interviewers, regular

calibration of instruments, random assignment of interviewers and multiple recall interviews from the same respondent. In addition, we collected duplicate diets of participants' food intake at the preschool, for laboratory analysis, which improved the accuracy of vitamin A intake estimations during the first round of dietary intake assessments. However, these measures do not fully eliminate errors attributable to recall bias and consequently, nutrient intake estimations.

Based on the large variation in reported studies on beta-carotene bioconversion studies [11,16–19], we used a conservative factor of 7:1 for the bioconversion of beta-carotene to retinol in biofortified cassava , which is higher than the 12:1 ratio recommended by the Institute of Medicine's (IOM) for mixed diets, yet, lower than the 3:7:1 ratio reported in a study on yellow cassava in gerbils [20]. Our conclusion on this is that a conservative factor was more appropriate to prevent an overestimation of vitamin A intake from cassava.

### D. Effect Modification & Confounding

A confounder is an extraneous variable that is related to a study's independent and dependent variable, and is also causally related to the dependent variable, whereas, in an effect modification, the effect of an exposure on an outcome is modified or different for varying levels of a third variable

#### (i) Effect modification in the randomized controlled trial

In the efficacy trial **(chapter 2)**, the possibility of introducing bias to the study based on the initial vitamin A status of participants. was addressed by using a stratified block randomization design [21]. This technique was implemented by categorizing participants into three different strata (terciles) corresponding to the lowest, medium and highest serum concentration of retinol binding protein. Participants were thus randomly selected systematically from each stratum into the yellow cassava and white cassava groups, such that both intervention and control groups had an equal representation of participants from all three RBP strata.

Another possible effect modifier was the high prevalence of inflammation and malaria observed in the study population, since serum retinol and retinol binding protein concentrations are decreased during inflammation[9,22,23] as well as malaria [24–26]. To reduce this potential bias, we dewormed all children two weeks before study commencement to reduce the possible effect of helminthic infections on serum retinol. Similarly, we tested all children and treated infected children for malaria prior to the commencement of the intervention.

**(ii) Effect modification/confounding in the observational studies**

While estimating the contribution of yellow cassava to vitamin A intake **(chapter 4)**, palm oil intake was a possible effect modifier. For example, since palm oil consumption was high in the community, those who consumed more palm oil at home could have biased our estimations of RAE intake. However, we specifically addressed this, by measuring the palm oil intake in the households of the children during the food intake assessment. In addition, we simulated palm oil retention in household dishes by accounting for cooking practices such as palm oil preheating and frying. This enabled us to estimate the intake of provitamin A from palm oil of participants in the households and to adjust appropriately while estimating the vitamin A contribution from other food sources.

In chapter five, while studying the effect of malaria on retinol binding protein, inflammation status was identified as another effect modifier. For example, malaria infection causes inflammation, and during inflammation serum retinol binding protein concentration is reduced. In controlling for this, we compared groups with zero malaria parasitaemia and no inflammation with other groups with varying intensities of malaria and/or inflammation. However, this led to a loss of sample size as well as power, which possibly biased our result.

Furthermore, in estimating the effect of inflammation and/or malaria on serum RBP **(chapter 5),** low intake of vitamin A could confound serum RBP. For example, low intakes of vitamin A in participants could have led to low serum RBP. At the same time, low vitamin A intake could have increased susceptibility to infection or inflammation due to lower immunity.

**External Validity**

External validity addresses the issues concerning the generalizability of a study [27]. Generalization is an act of reasoning that involves drawing broad conclusions from particular cases. This usually entails making an inference about an unobserved population based on an observed one [28]. In the context of the efficacy trial with biofortified cassava, the interest would be to apply the findings to a population where deficiency of vitamin A is most prevalent – a population of under-five preschool children [29,30]. In summary, external validity should answer the question: *would the consumption of biofortified (yellow) cassava increase serum retinol concentration in Nigerian (or other) children when consumed for the same or longer period?*

To a large extent, we think the result of the efficacy trial reported in chapter two are generalizable. This is because the study was conducted in pre-school children which represents the target population of under-five preschool children. Although, vitamin A deficiency was not highly prevalent

in the population, the effect of this as explained earlier would have most likely led to an underestimation of effect size, rather than an overestimation.

This study was in many ways like the efficacy trial conducted by Talsma *et al* in 2015 in a Kenyan population of older children. Although, the effect of consuming biofortified cassava on Kenyan and Nigerian children are similar, the effect on serum beta carotene was different between the two populations. The differences observed in these two populations is probably attributable to the high palm oil intake in the Nigerian population evident by the very high serum beta-carotene levels at baseline. [31].

With regard to the contribution of biofortified cassava to vitamin A intake **(chapter 4)**, the robust and rigorous dietary assessment approach used, supports the generalizability of the study in many ways. First and foremost, is the conservative beta-carotene/retinol bioconversion ratio used for biofortified cassava foods, as earlier mentioned. Secondly, we carefully accounted for palm oil intake outside the pre-school during the dietary intake assessments. Thirdly, we measured dietary vitamin A intake during and after the intervention and used a more robust statistical method to account for inter-individual differences in retinol intake during and after the intervention. These approaches were taken to minimize possible threats to external validity, which increases the chances for generalizability in a different population.

**Public Health Importance and Future studies**

In this thesis, we confirm that the consumption of yellow cassava improves serum retinol. This is the second efficacy trial conducted on biofortified cassava, and our results confirm that the consumption of biofortified cassava is efficacious, and when consumed over time, can increase serum vitamin A concentration modestly.

The goal of the biofortification programme is to contribute to reducing the high prevalence of specific micronutrient deficiencies, especially iron, zinc and vitamin A, by producing micronutrient-dense staple foods. Compared to other strategies for tackling micronutrient deficiencies, biofortification remains the most sustainable and cost-effective. Furthermore, the possibility of sharing micronutrient-dense germplasm makes biofortified foods available and accessible especially in rural areas, where staple foods are usually produced and consumed.

Although this study focused on demonstrating efficacy in pre-school children, biofortification of provitamin A foods can contribute to body stores of vitamin A in all age groups, throughout the life cycle. Children 6-23 months who are still breastfeeding can also benefit indirectly through

maternal consumption of provitamin A biofortified staple foods, which can increase breast milk concentrations of vitamin A.

Due to the limitations encountered in this study, it was impossible to assess liver stores among participants. It would therefore still be useful for future research to focus on this. In addition, it is still not very clear if liver stores are affected during inflammation; this can also be answered in future studies. Furthermore, there are still unanswered questions concerning beta-carotene metabolism and bioconversion, and single nucleotide polymorphisms (SNPs) associated with beta-carotene bioconversion. Genetic studies targeted at candidate genes may also be useful in identifying SNPs specific to the Nigerian/African population. Finally, more isotope studies are also needed to study the bioconversion of beta-carotene in different food matrices foods.

Meanwhile, in implementing the delivery phase of the biofortification programme, emphasis should be put in promoting diverse diets to ensure the adequate nutrition of the larger population.

**Conclusion**

In conclusion, biofortified (yellow) cassava efficaciously improved serum retinol concentrations in preschool Nigerian children and can potentially contribute up to 63% of estimated average requirement for vitamin A intake in the Nigerian population. Malaria and inflammation are still important determinants of serum retinol concentration and can jointly contribute to vitamin A status.

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# English Summary

**English Summary:**

Vitamin A deficiency (VAD) is still a micronutrient deficiency of public health significance in low income countries. Biofortified provitamin A-rich cassava can potentially alleviate vitamin A deficiency, and can be used along with other dietary approaches to improve the vitamin A status of vulnerable populations.

**Chapter 1** provides a layout of the rationale and objectives of the PhD research as well as a general introduction of the problem of VAD, followed by interventions to address the problem, while focusing on the role of biofortification and the long term strategies of biofortification. It also presents issues around bioavailabilty, bioconversion and transport of vitamin A in the body, and the isotope dilution methodology used in the assessment of total body vitamin A pool. The chapter is concluded with the geographical description of the study area.

**Chapter 2** describes the contribution of pro-vitamin A biofortified cassava to serum retinol among preschool children who consumed foods prepared with yellow cassava. The study was an open label randomized controlled efficacy trial conducted in a south-west Nigerian community. The primary objective was to determine the efficacy of biofortified cassava to improve retinol concentrations of Nigerian preschool children, while the secondary objective was to test if there was difference in gut permeability between the experimental and control groups. 176 pre-school children (3-5 years old) were randomized into two parallel arms comprising an experimental group (n=88), fed foods prepared from biofortified cassava, and a control group (n=88), fed foods prepared from white cassava, twice a day, six days a week for 93 days. The intervention increased serum retinol in the experimental group by 0.06 μmol/L (95% CI: 0.004, 0.124). No significant treatment effects were detected for serum β-carotene (adjusted effect: 3.9%; 95% CI: -0.6%, 8.6%) and gut permeability (adjusted effect: 0.002 (95% CI: -0.089, 0.092), but a significant effect was detected for hemoglobin concentrations (adjusted effect: 3.08 g/L (95% CI: 0.38, 5.78).

In **chapter 3**, the effect of urinary volume on small intestine permeability assessment is discussed. This report is based on the phenomenon observed during the study on gut permeability assessment among the children. In this study, urinary volume per kilogram body weight per hour excreted over a 90-minute period after administration of a sugar solution, was measured, and the differences in sugar ratios were compared between children with high and low urinary volumes, as well as between children who voided and who were unable to void before administration of the test solution. It was observed that sugar permeability ratios varied significantly with total urinary volume during the sugar permeability tests. Lactulose-

mannitol ratio ($p<0.001$), lactulose-rhamnose ratio ($p<0.001$), and sucrose-rhamnose ratio ($p<0.002$) were all significantly higher in children who voided lower volumes of urine compared with children who voided higher volumes of urine.

Having shown that the daily consumption of yellow cassava improved serum retinol concentration, **chapter 4** attempted to answer the question of the contribution of biofortified cassava to total dietary intake. Dietary intake was assessed as part of the same randomized controlled trial reported in chapter two. Intake assessments were conducted during the intervention and one month after, when children had resumed their habitual diet. Differences in RAE intake between groups and the two time points were compared using a linear mixed regression analysis. The study showed that the median RAE intake was higher in the yellow cassava group, compared to the white cassava group. Children consumed a median amount of 536 µg/day and 301 µg/day in the yellow cassava and white cassava groups respectively during the intervention. After the intervention, RAE intake was 300 µg/day and did not differ between groups ($p=0.5$). The study showed that if white cassava replaced yellow cassava, the potential contribution of yellow cassava to total RAE intake would be about 32%.

Being a malaria and inflammation endemic population, it was important to study the contributions of malaria and infection and inflammation to serum retinol concentrations. **Chapter 5** compared existing adjustment methods used for correcting serum retinol concentration during infection. A secondary objective as well, was to study the effect of malaria infection and inflammation on serum retinol concentrations among 427 children who participated in the screening exercise before the commencement of the efficacy trial. Serum RBP, C-reactive protein and α-1-glycoprotein were analysed using a sandwich ELISA technique, malaria parasitaemia was measured using a real-time quantitative PCR. Participants were categorized into no parasitaemia, low/moderate parasitaemia and high parasitaemia. Adjusted RBP concentrations were calculated by various methods that are commonly used for the adjustment of serum retinol concentration, here referred to as the Thurnham, the BRINDA and the Palmer correctional methods. The results showed that children with no inflammation but with low/moderate parasitaemia had a lower mean RBP concentration ($0.89 \pm 0.27$ µmol/L; $p=0.04$) compared with children with no inflammation and no parasitaemia, ($0.98 \pm 0.27$ µmol/L; $p=0.04$). The Thurnham and Palmer adjustment methods gave similar estimates of adjusted RBP (0.95 µmol/L ± 0.28 µmol/L and 0.96 µmol/L ± 0.28 µmol/L, respectively), while the BRINDA method gave a higher estimate of $1.03 \pm 0.3$ µmol/L ($p<0.0001$).

Finally, **chapter 6** discusses the main findings and conclusions of this thesis, while highlighting the public health perspective of the thesis and providing recommendations for future studies..

Overall, we can conclude that yellow cassava is indeed efficacious in improving serum retinol concentrations in preschool Nigerian children, and in combination with other dietary diversification approaches, can be used to attain nutrient adequacy in deficient populations, especially children populations.

# Acknowledgement

**Acknowledgement**

Writing an acknowledgement is always a tricky task, as one always runs a risk of forgetting that crucial person, who should not have been forgotten. If while reading this, you discover that you are that person, I apologize in advance and dedicate this paragraph to you.

Cassavita II project, as we informally called it, was a project filled with lots of intrigues and suspense. It was a project in Telemu (Osun State, Nigeria), that cannot be quickly forgotten, because of the often-dramatic events that spanned the project from beginning till the end. It was a project filled with numerous unforgettable memories and experiences of people and events. The experiences for me, were mostly new, as it was my first direct encounter with rural life. It happened that some of these experiences were not all rosy, however, for me, they were probably more important lessons than the science that formed the core of my Ph.D. journey. They were lessons that needed to be learned about life and people. Perhaps more importantly, they were lessons that I needed to learn about myself and how I perceived my environment. For the first time in my life, I was handling a project with such a broad scope that, in retrospect, if I was clearly informed about what I was getting myself into from the beginning, I might have looked elsewhere for a less complicated PhD programme.

Conceptualizing and designing a feeding trial in Nigeria may be an easy task on paper. However, implementing a project of such magnitude successfully, with all the scientific rigours entailed, was an arduous task that required a strenuous effort from beginning till the end. We had to conduct a census, recruit participants, set up a pre-school, employ and manage teachers, educate two hundred and fifty children, feed them, and generously provide health care. All these happened in a rural setting, where the expected minimum education and health facilities were hardly accessible to the community. Our peculiar situation highlighted the difficulty of implementing practically any project in Nigeria. Therefore, the most important subject of my acknowledgement is God, who I consider a principal component of the project, without whom we may not have been successful on the field, considering all the seemingly unsurmountable challenges we encountered.

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# About the Author

Curriculum Vitae

List of publications

Overview of completed activities

**Curriculum Vitae**

Ibukun Afolami was born on the 3rd of November, 1983 in Abeokuta, Ogun state, Nigeria, where he received primary and secondary education at St. Bernadette's Nursery & Primary School and African Church Grammar School respectively. In 2003 he got admission into the University of Lagos to study Biochemistry. During this period, his interest in science began to grow rapidly. He completed his bachelor's programme in 2008 and Between 2008 and 2009, underwent a compulsory National Youth Service Program (NYSC).

In 2011, after obtaining a master's degree in Biochemistry at the University of Ibadan, he ventured into Human Nutrition in a quest to apply his basic science knowledge in this discipline.

Ibukun is a fellow of the prestigious African Nutrition Leadership Programme (ANLP), and a beneficiary of the HarvestPlus/IFPRI/CIAT multi-partner-funded PhD fellowship in Wageningen University in the Netherlands.

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**Afolami, I**., Samuel, F., Borgonjen-van den Berg, K., Mwangi, M.N., Kalejaiye, O., Sanusi, R.A., Putri, L.A.R., Brivio, F., Brouwer, I.D. and Melse-Boonstra, A. (2021). The contribution of provitamin A biofortified cassava to vitamin A intake in Nigerian pre-schoolchildren. *The British Journal of Nutrition*. 1–9.

**Afolami, I**., Mwangi, M.N., Samuel, F., Boy, E., Ilona, P., Talsma, E.F., Feskens, E. and Melse-Boonstra, A. (2021). Daily consumption of pro-vitamin A biofortified (yellow) cassava improves serum retinol concentrations in preschool children in Nigeria: a randomized controlled trial. *The American Journal of Clinical Nutrition*. 113.1: 221–231.

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**Overview of completed training activities**

**Discipline-specific courses and activities**

| Name | Organizer & Location | Year |
|---|---|---|
| African Nutrition Leadership Program ANLP | North West University, Potchefstroom, SA. | 2013 |
| African Nutrition Epidemiology Conference (ANEC) | Accra, GH | 2014 |
| Laboratory training on CRP and RBP analysis | HNH-WUR, Wageningen, NL | 2015 |
| Health and Sustainable Diets | VLAG, Wageningen, NL | 2018 |
| Energy Metabolism and Body Composition in Nutrition and Health Research | VLAG, Wageningen, NL | 2018 |
| Philosophy of Science | Studium Generale, NG | 2019 |
| Philosophy of Nature | Studium Generale, NG | 2019 |
| Introduction to Philosophy | Studium Generale, NG | 2019 |
| Doctors as Educators | West African College of Physicians, Ibadan, NG | 2014 |
| Research Design & Methodology for Doctoral Students | University of Ibadan/National Institute of Health/ National Institute of Child Health and Human Development, Ibadan, NG. | 2014 |
| Pedagogy of Higher Learning | University of Ibadan/National Institute of Health/ National Institute of Child Health and Human Development, Ibadan, NG. | 2014 |

**General Courses**

| Name | Organizer & Location | Year |
|---|---|---|
| VLAG PhD Week | VLAG, Wageningen, NL. | 2015 |
| Introduction to Digital Library | WUR library, Wageningen, NL | 2015 |
| Introduction to Mendeley | WUR library, Wageningen, NL | 2015 |
| The Essentials of Scientific Writing and Presenting | WGS, Wageningen, NL | 2015 |
| Brain Training | WGS, Wageningen, NL | 2018 |
| Logic I | Studium Generale, NG | 2015 |
| Logic II | Studium Generale, NG | 2015 |

**Optional**

| Name | Organizer & Location | Year |
|---|---|---|
| Preparing PhD Research Proposal | HNH-WUR, Wageningen, NL | 2018 |
| Applied Data Analysis in Human Nutrition & Research (HNE37306) | HNH-WUR, Wageningen, NL | 2018 |

**Colophon**

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