

Yellow Cassava: Efficacy of provitamin A rich cassava on improvement of vitamin A status in Kenyan schoolchildren

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Thesis

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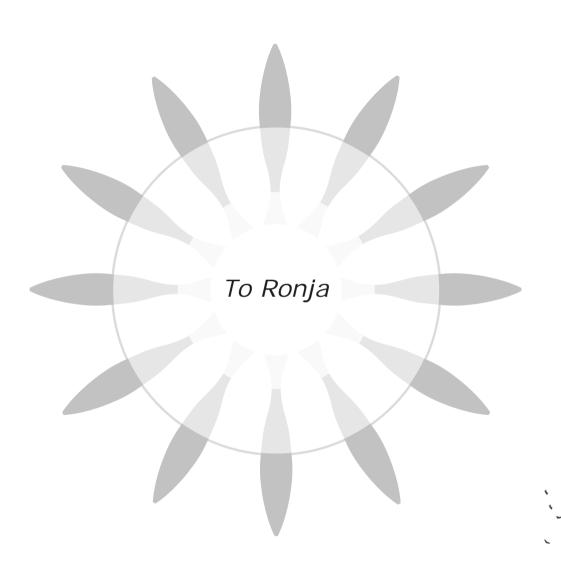
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Abstract

Background: Biofortified yellow cassava has great potential to alleviate vitamin A deficiency in sub-Saharan Africa and can be used as a complementary approach to other interventions. However, direct evidence whether yellow cassava can significantly contribute to the vitamin A intake and status of populations is required. The overall aim of this thesis is to provide proof of principle whether biofortified yellow cassava can improve the vitamin A status of schoolchildren in Kenya.

Methods: The research was conducted in Kibwezi district, Eastern Kenya. First the effect of daily consumption of yellow cassava was assessed in 342 primary school children in Kenya in a randomized controlled feeding trial with serum retinol concentration as primary outcome. Furthermore we investigated the sensory and cultural acceptability of yellow cassava in a cross-sectional study (n=140) in three primary schools for children as well as their caretakers. Next we studied the diagnostic performance of several proxy markers to assess vitamin A deficiency in comparison with serum retinol concentration as a field based method to assess vitamin A deficiency (n=375). And last we used the dietary intake data of children in the randomized controlled trial to model the potential contribution of yellow cassava to the nutrient adequacy of micronutrient intake using linear programming.

Results: The randomized controlled feeding trial collected complete data for 337 children with a compliance of 100%. Primary analyses (per protocol) showed that serum retinol concentrations in the yellow cassava group, increased with 0.04 µmol/L (95%CI: 0.00-0.07 µmol/L) compared to the white cassava group and secondary analyses showed that serum β-carotene concentration increased with 524% (448%-608%). No evidence of effect modification by initial vitamin A status, zinc status, or polymorphisms in the β -carotene monooxygenase gene was found. In the acceptability study 72% of caretakers and children were able to detect a significant difference in taste between white and yellow cassava and indicated to prefer yellow cassava because of its soft texture, sweet taste and attractive color. Serum concentrations of retinol binding protein, transthyretin and C-reactive protein combined showed excellent diagnostic performance in estimating vitamin A deficiency in primary school children, with an area under the curve of 0.98. Adding yellow cassava to the diet as a school lunch improved the nutrient adequacy of the diet of schoolchildren, however, even with the addition of nutrient dense foods such as fish and oil, nutrient adequacy could not be ensured for fat, riboflavin, niacin, folate and vitamin A.

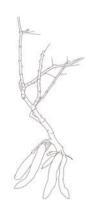
Conclusions: Consumption of yellow cassava is acceptable and improves the serum retinol concentrations of primary school children in Kenya. The combination of three proxy markers is a promising approach to measure vitamin A deficiency in a low resource setting. Yellow cassava contributes to a better nutrient adequacy but should be accompanied by additional dietary guidelines and interventions to fill the remaining nutrient gaps.

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Chapter 1

General Introduction



Introduction

Biofortified yellow cassava has great potential to alleviate vitamin A deficiency in sub-Saharan Africa and can be used as a complementary approach to other interventions^[1]. However, direct evidence whether yellow cassava can significantly contribute to the vitamin A intake and status of populations is lacking. The research described in this thesis is conducted in the early developmental phase of biofortified yellow cassava and should be seen as proof of principle to support further development of the crop. For this, we conducted an efficacy study with yellow cassava to assess its capability to improve vitamin A status of schoolchildren in Eastern Kenya. This research forms an important step to provide evidence to governments and organizations for endorsement and effective targeted dissemination and delivery of this new crop.

Vitamin A deficiency is a major public health problem among vulnerable groups of young children and pregnant women in low-income countries^[2]. Deficiency of vitamin A leads to impaired vision and reduced immunity, and compromises growth and development leading to death in the most severe cases^[3]. In 2011, an estimated number of 157,000 of global child deaths were attributable to vitamin A deficiency^[4]. The main cause of vitamin A deficiency is a low dietary intake of bioavailable vitamin A. Therefore, strategies to increase the supply of vitamin A to the vulnerable groups receive high priority. Supplementation programs with vitamin A for children under 5 years of age are an important strategy to reduce vitamin A deficiencies. Vitamin A supplementation in the form of two high doses per year proved to reduce mortality by 24%^[5,6,7]. However these programs often struggle with coverage, specifically failing to reach those living in the most rural areas^[8]. Also the WHO indicates in its advise concerning vitamin A supplementation that the intervention should go along with other strategies to improve vitamin A intake^[9].

Food-based strategies are recommended as promising approaches to meet vitamin A needs^[10] and the benefits include not only intakes of specific nutrients but also improved diet and health status. Food-based approaches comprise dietary diversification, fortification and biofortification. The monotonous, cereal-based diets generally consumed in developing countries urge for better dietary diversity. However, effective diversification of diets for improved vitamin A status would inevitably involve animal food sources such as meat, which is generally expensive and unavailable in rural communities^[11]. Promoting plant-based foods with the purpose to improve vitamin A status should be done carefully as studies indicate that efficacy is disappointing probably due to low bioavailability of vitamin A^[12,13,14,15]. Food fortification programs form another strategy to reduce vitamin A deficiency and fortified oil and sugar have shown to be effective in Central-America^[16]. However it is questionable whether these products are affordable or commercially available for those most in need^[17].

In Kenya, voluntarily fortified oil and sugar with vitamin A are available on the market since 2012. Coverage of vitamin A supplementation among children 6-59 months (having received a dose of vitamin A in the past six months) in Kenya was estimated at only 30% by the 2008-2009 Demographic and Health Survey^[18] and at 31% in a recent survey in 2012^[19]. Hence, the majority of the children are not being reached, and more than 60% of Kenyan preschool children still suffer from moderate or severe vitamin A deficiency^[2]. This urges for the development of additional interventions to fill the gap in the existing strategies.

Biofortification

Biofortification is the process of breeding nutrients into food crops, either by using natural breeding techniques or by using genetically modified organisms. For some crops wild varieties are available having specific nutrient producing traits than can be used for breeding. For other crops these traits are not present and the only way of changing the nutrient profile of the crop is by genetic engineering. Although biofortified staple crops contain only small amounts of micronutrients, frequent consumption in large quantities throughout the year will provide a steady supply of micronutrients^[1,20]. Biofortification is considered to be sustainable because it requires a onetime investment only that further allows farmers to continuously grow the micronutrient rich planting material for years to come, at no more cost than the regular crop would cost. In addition, biofortified crops can reach poor people that often rely on their own produce of staple crops as their main source of food, and who live in rural areas where supplementation programs cannot reach or who cannot afford fortified products^[17]. Therefore, biofortification is a promising additional strategy to increase the daily adequacy of vitamin A intake.

HarvestPlus is part of the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH) that promotes biofortification through natural breeding processes (www.harvestplus.org) and streamlines careful planned research on new biofortified crops. HarvestPlus has laid out the following steps with questions to quide the development of biofortified crops (Figure 1):

Step 1: Discovery: Can breeding increase the micronutrient density in food staples to concentrations that will likely have an impact?

Step 2: Development: Is the crop able to improve the micronutrient status under controlled conditions? Are the crops acceptable by the farmers and communities for consumption?

Step 3: Delivery: Is the crop grown by farmers and eaten by consumers after being officially released and promoted by communication campaigns? Does the crop improve the micronutrient status of the target population?

These steps are addressed by a collaborative effort of plant breeders, nutritionists, farmer ecologists, sociologists and economists. The first step can be answered when information is available about the current dietary food patterns and methods of preparation, in order to calculate to what minimum level the micronutrient in the crop should be bred. The second step can be assessed by efficacy trials preferably conducted in the target population that is in need of the micronutrient and by acceptability studies of both farmers and consumers. The third step can be addressed once the crop is released to farmers and communities by effectiveness trials that involve extensive communication strategies to maximize the impact of the crop. This provides a useful framework for the successful development and introduction of biofortified crops.

Some examples of biofortified staple crops are high zinc rice and wheat; provitamin A rich maize, cassava and orange fleshed sweet potato; and high iron beans and pearl millet^[17]. One of the successful biofortified crops that have been introduced already in Africa is the orange fleshed sweet potato. Varieties with high concentration of carotenoids (5,023-11,278 μ g total β -carotene per 100 g fresh weight)^[21] have proven to increase the vitamin A status in children in South Africa^[22], Mozambique and Uganda^[23,24]. Effectiveness trials in Mozambique and

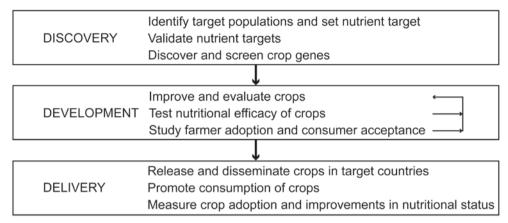


Figure 1: Steps in the development of biofortified crops (adapted from Saltzman *et al.* 2012) $^{[17]}$

Uganda showed that the adoption of the crop by both farmers and consumers was excellent given that 77% of consumers were cultivating the crop themselves and this resulted in a 42-169% increased intake of vitamin A^[21]. Orange fleshed sweet potato is currently available in many African countries and contributes to reducing the prevalence of vitamin A deficiency. Although evidence for efficacy and effectiveness for orange fleshed sweet potato is nearly complete, such evidence is still lacking for most of the other newly developed biofortified crops.

Cassava

Cassava (*Manihot esculenta* Crantz) originates from South America and is believed to be brought to Africa in the 17th century. The roots of cassava became a staple food for many people since the late 19th and early 20th century^[25]. The roots are predominantly consumed but also the leaves are edible. Cassava is often referred to as a food security crop because of several reasons; firstly, the planting and harvesting time is flexible, which allows it to be harvested when really needed; secondly, because of the tolerance towards low soil fertility and water availability; and thirdly, the plants can be multiplied by vegetative propagation without the need for seeds, allowing farmers to continue to grow the crop without financial input^[25]. Cassava cultivation has increased over the past three decades in Eastern Africa due to increasing land pressure by population growth and also due to providing income to farmers^[26]. This makes cassava an important staple crop in deprived areas.

A disadvantage of cassava is the short shelf life of the root after harvesting due to a rapid physiological deterioration process within the root that causes inedible blue and black pigments^[27]. Therefore, cassava has to be processed or consumed within 24-36 hours after harvesting. Another disadvantage is that cassava contains unhealthy cyanogenic glucosides in variable concentrations depending on the variety. A variety is called sweet when the cassava contains only a small amount of cyanogenic glucosides (<100 mg/kg fresh weight) and can then be consumed fresh as boiled cassava. Varieties with higher amounts of cyanogenic glucosides (100-500 mg/kg fresh weight) are called "bitter" and need to be

processed before consumption by for example drying, fermenting or grinding after which the flour is baked or fried^[28]. In West-Africa cassava is mostly consumed after it has been crushed and fermented for 3-10 days followed by roasting into a granola type of flour which is used for preparing certain dishes. In Kenya, cassava is either consumed as a dish made from cassava flour after fermenting or drying of the cassava, or as freshly boiled root chunks. A third disadvantage is the low nutrient density with starch as the main component. Per 100 g raw weight, cassava provides 580 kJ of energy, a little protein(1.3 g) and fat (0.6 g) and traces of iron (0.9 mg), niacin (0.9 mg), thiamin (0.06 mg), riboflavin (0.05 mg), calcium (33 mg), zinc (0.3 mg) and vitamin C (31 mg)^[29]. A food consumption survey in 2-5 year old children in Western Kenya^[30] showed that 59% of their energy intake comes from cassava. The low density of nutrients in cassava therefore poses a risk for inadequate vitamin A, zinc and iron intake in children relying on cassava as their staple food.

Yellow cassava

Varieties of cassava naturally rich in provitamin A, and therefore yellow colored, were encountered in the Amazon region in South America. These varieties were used by the International Centre for Tropical Agriculture in Colombia to develop varieties with higher β -carotene concentrations through natural plant-breeding techniques $^{[31,32]}$ and have been introduced into Africa by the International Institute of Tropical Agriculture (IITA) in Nigeria. Yellow cassava was introduced in Kenya in 2008 for the purpose of the research described in this thesis and is currently grown experimentally by the Kenya Agricultural Research Institute (KARI) for adaptation and cross breeding with local varieties.

Total carotenoid concentration in fresh yellow cassava ranges from 1–100 µg/g (fresh weight), primarily as all-trans- β -carotene[$^{[32]}$ and is located in the parenchyma cells, the storage cells of the roots[$^{[31]}$. Yellow cassava varieties that have recently been released in Nigeria (2011) contain ~ 6 µg/g (fresh weight) total β -carotene, while breeding is on-going to deliver varieties up to 15 µg/g (fresh weight)[$^{17]}$. The color of the crop ranges from deep yellow to slightly orange. Carotenoid concentration is a stable trait and is influenced more by genotype than by its environment[$^{[33]}$. Studies showed that retention of carotenoids differs not only per processing and storage method for a certain variety[$^{[34]}$ but also within a variety and this might be due to the variable distribution of dry weight matter within a root[$^{[35]}$. Retention varies between 10% for heavily processed and roasted cassava granules[$^{[36]}$ to 87% for boiling[$^{[35]}$.

Although yellow cassava is an excellent source of β -carotene and energy, it is known to be generally poor in other nutrients such as iron and zinc[37]. Children whose diets consist largely of cassava, even when yellow, may still be vulnerable to micronutrient deficiencies[30,38]. Therefore, the introduction of yellow cassava, may require additional food-based dietary guidelines to fill the existing gap in nutrients in the diet.

Acceptability, one of the components of the development of a biofortified crop, has not been studied for yellow cassava^[1]. The appearance and taste of the yellow variety are different than the white variety, partly because of the difference in dry matter content which behaves differently during preparation^[34]. Several agronomic traits like yield and resistance to pests might also be different for yellow cassava, although yellow varieties showed a delay in the onset off post-harvest



deterioration which can be helpful in the acceptance of the crop for both farmers and consumers^[32].

Overall, cassava as a staple crop has advantageous agronomic qualities, but contains low amounts of micronutrients. Yellow cassava seems a promising crop, yet it needs to be proven whether yellow cassava is acceptable to future consumers and whether it can significantly contribute to vitamin A intake and status of populations in need. Furthermore we need to explore the effect of consumption of yellow cassava on the potential adequacy of intake of other micronutrients.

Vitamin A

Absorption and transport

A detailed picture of the uptake and metabolism of retinol and β -carotene in the lumen can be found in figure 2. In summary, the small intestine is the primary site of uptake of retinol and carotenoids. After consumption of foods with retinol and carotenoids, these compounds are released from their food matrixes in the lumen and emulsified with dietary fatty acids and bile acids, making them more soluble as required for uptake into the enterocytes. In the enterocytes retinol is transformed to retinyl-ester and, as the carotenoids, packed with triglycerides and cholesterol into chylomicrons. Part of β -carotene from food will already be cleaved in the enterocyte by the β -carotene 15,15′-monooxygenase-enzyme (BCMO1) and transformed into retinal and further follow the same metabolic route as preformed retinol (Figure 3). After the formation of the chylomicrons they are released into circulation via the thoracic duct into the venous blood stream[39].

About 66-75% of dietary retinol and carotenoids in chylomicrons are taken up by the liver and stored in hepatic stellate cells. The remaining 25-33% is cleared by peripheral tissues during circulation and contributes to tissue retinol and carotenoid pools of humans. The BCM01-enzyme is also found in other cells than the enterocytes, such as in the liver, kidney, lungs, skin, testis, eye and embryonic tissue and contributes to the demand for retinol by the conversion of β -carotene into retinol in these cells^[40]. In the liver, retinol is stored in lipid droplets when there is sufficient or excess vitamin A present in the body and can be released for use when needed. Retinol binding protein is mainly produced in the liver, where it binds retinol before release into the bloodstream. Transthyretin is a large transport protein, with a high affinity for the retinol binding protein-retinol complex, that binds this complex after release from the liver. This transport complex delivers retinol to the peripheral tissues in the fasting state and might also prevent filtration of the small retinol binding protein-retinol complex by the kidneys^[41,42].

In summary, after absorption in the small intestine, β -carotene is directly incorporated into chylomicrons in the enterocyte, or converted into retinol before incorporation in chylomicrons, followed by release of chylomicrons in the blood stream for transport to respective tissues or to the liver for storage. After storage in the liver, retinol is transported in the blood within the retinol binding protein - transthyretin complex.

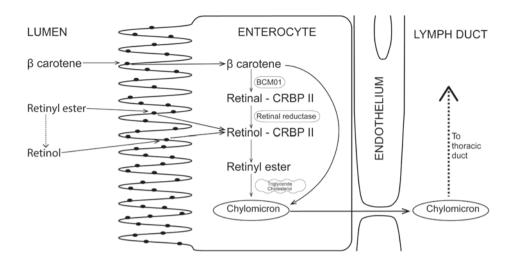


Figure 2: Uptake and metabolism of vitamin A and carotenoids (Adapted from D'Ambrosio *et al*, 2011)^[40]. CRBP II: Cellular retinol binding protein type II; BCMO1: β-carotene 15,15'-monooxygenase

Bioavailability and conversion

Vitamin A is an essential nutrient that is consumed from the diet either as preformed retinol which is readily absorbed from foods such as eggs and meat, or as carotenoids from yellow and green fruits and vegetables which requires conversion into retinol in the body. Examples of carotenoids with provitamin A activity are all-trans- β -carotene, cis- β -carotene, a-carotene and β -cryptoxanthin, but vitamin A activity is different for every carotenoid. All-trans β -carotene is the most abundant pro-vitamin present in food and is more efficiently converted to retinol in comparison to the other carotenoids. The vitamin A equivalency of dietary carotenoids is expressed as retinol activity equivalents: from a mixed diet, 12 μ g of all-trans- β -carotene or 24 μ g of other provitamin A carotenoids (a-carotene, cis β -carotene, β -cryptoxanthin) is converted into 1 μ g of retinol $^{[43,44]}$. These are estimations, and the bioavailability and conversion of carotenoids from foods into retinol depends on both food- and host-related factors $^{[14,45]}$.

An important food related factor is the matrix in which the carotenoids are embedded; in green, photosynthetic plant tissue, carotenoids are entrapped within chlorophylls in pigment-protein complexes in the thylakoid membranes of chloroplasts [46]. For example, processing of spinach as a means to destruct the food matrix before consumption, increased the bioavailability of β -carotene in humans [47]. A study in Indonesian children showed that carotenoids from orange fruits are more bioavailable than those from green vegetables [12]. Food matrix issues are likely of less concern with yellow cassava, as the β -carotene is stored in parenchyma cells which can probably easily be destructed in the human gastrointestinal tract. This was also shown by a study with yellow cassava in gerbils that showed a bioconversion factor of 3.7 μ g β -carotene to 1 μ g retinol [48]. Another study in 10 American women showed a conversion factor of 4.2 μ g β -carotene to 1 μ g



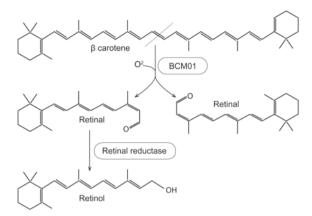


Figure 3: Conversion of β-carotene into retinol (adapted from von Lintig, 2012)^[41]. BCMO1: β-carotene 15,15'-monooxygenase

retinol^[49], but inter-individual variation was high (range 0.3 to 10.6). Another limiting factor for bioavailability of carotenoids is the amount of fat available in the food; however only 3-5 g of fat is estimated to be sufficient for efficient absorption of physiological amounts of β -carotene^[50,51]. In yellow cassava, the conversion of β -carotene seems to be more an issue of host-related factors than of food-related factors, and needs to be further explored.

Vitamin A status of the host is an important factor that influences the conversion of carotenoids, as was seen in a 3-day study with fruit and vegetables in Filipino children that showed the highest intervention effect in children who were vitamin A deficient[52]. Mouse studies showed a feedback mechanism for the conversion of β-carotene by the BCM01 enzyme, which was up-regulated during deficiency and down regulated during sufficiency. Although this has not been shown in humans yet, it can explain why extensive β-carotene supplementation never leads to hypervitaminosis of vitamin A^[40], whereas retinol supplementation does^[53]. Carotenoids may also be metabolized differently in persons that harbor certain genetic polymorphisms, for example in the β-carotene 15,15'-monooxygenase (BCMO1) gene, which encodes the enzyme responsible for the central cleavage of B-carotene into retinol^[54]. A few studies have shown the presence of common single nucleotide polymorphisms (SNP) in this gene leading to lower bioconversion of β-carotene to retinol in Caucasians^[55,56]. Whether polymorphisms also play a role in the conversion of β-carotene into retinol in the genetically divers African population is currently unknown.

Measuring vitamin A status

Vitamin A is stored primarily in the liver as retinol and therefore liver stores reflect the true vitamin A status. However, direct measurement of liver stores is not possible for obvious reasons. Serum retinol concentration is considered to be the reference method for assessment of vitamin A deficiency $^{[2,57]}$. Children are considered to be deficient when retinol concentrations fall below 0.7 μ mol/L and severely deficient when below 0.35 μ mol/L. In this concentration range, serum retinol is linearly associated with hepatic stores. However at concentrations above 1.05 μ mol/L retinol is homeostatically regulated by the liver and reflects the sufficient

state. Therefore the degree of sufficiency cannot be measured with serum retinol^[58]. Another limitation of serum retinol concentration is that it is influenced by inflammation because its transporter protein, retinol binding protein reacts as a negative acute phase protein: it is transiently decreased by infection-induced inflammation but typically returns to pre-infection levels within a few days^[59].

The optimal method for measuring retinol concentration is by high-performance liquid chromatography (HPLC), which is expensive, technically demanding and rarely available in developing countries[60]. In addition, measurement of serum retinol requires large serum volumes (0.5 mL) that can only be obtained by venipuncture, and must be stored in tubes impermeable to light until laboratory analysis. Stable isotopes dilution techniques have been validated and used as a proxy to measure the body vitamin A pool and liver stores[15]. This has the advantage that vitamin A status can be measured over the full range, and response to treatment is larger which considerably reduces the sample size needed for such studies. However this method is logistically even more challenging as it requires more expensive equipment and technical expertise than the measurement of retinol, and is even more scarcely available in resource poor settings. Retinol binding protein, the transporter protein of retinol in blood is sometimes used as a proxy indicator of vitamin A status^[60]. However, quantification of retinol binding protein differs per assay which makes it difficult to define universal cut-offs defining deficiency. In addition, also the molar ratio of retinol binding protein to transthyretin has been used as an indicator of vitamin A status which would be unaffected by inflammation^[61]. As an alternative to measuring serum retinol by HPLC, rapid in-field measurement by fluorometry is under development, exploiting the characteristic of retinol to fluoresce under influence of ultraviolet light, particularly when bound to RBP[62]. However this assay method has not yet been validated against the reference method.

For now, retinol concentration measured by HPLC remains the best indicator for measuring retinol in children who are deficient, but there is need for easy to use field methods that can be applied in resource poor settings to measure vitamin A status.

Rationale and objectives

Vitamin A deficiency is a major public health problem among vulnerable groups of young children and pregnant women in low-income countries. In Kenya, despite supplementation and food fortification programs, more than 60% of preschool children still suffer from vitamin A deficiency and this urges for additional complementary interventions. Biofortification of staple crops is a promising approach to reduce vitamin and mineral deficiencies in a sustainable way. However, evidence for efficacy and effectiveness is still lacking for most of the newly developed biofortified crops. Cassava is one of these staple crops for which a biofortified variety has been developed. The original, white, cassava has many agronomic advantages, but it contains very low amounts of essential micro-nutrients. Yellow cassava contains substantial amounts of B-carotene which may improve the vitamin A status of children. Up till now, neither acceptance by future consumers of yellow cassava has been proven, nor whether it can improve vitamin A status or what effect additional consumption of yellow cassava will have on the intake of other essential nutrients. Measuring vitamin A status in low resource settings is a challenge. Retinol concentration measured by HPLC is the reference indicator for



assessing vitamin A status in children who are deficient, but there is need for easy to use field method that is less costly and needs less infrastructure than HPLC.

The overall aim of this thesis is to provide proof of principle whether biofortified yellow cassava can improve the vitamin A status of children in Kenya. The following specific objectives were formulated:

- 1. To assess the efficacy of biofortified yellow cassava in improving serum retinol concentration of marginally deficient children
- 2. To assess the sensory and cultural acceptability of biofortified cassava
- 3. To assess the vitamin A deficiency prevalence of primary school children in Kibwezi district
- 4. To assess the diagnostic value of field methods for measuring vitamin A status in children in comparison with serum retinol concentration by HPLC
- 5. To assess whether the provision of yellow cassava as a school lunch can improve the dietary nutrient adequacy of primary school children

Outline of this thesis

This thesis is based on two cross sectional studies and one randomized controlled trial conducted from 2008 to 2012 in Kibwezi and Makindu district in Eastern Kenya in primary school children aged 5 to 13 years of age.

Chapter 2 describes a randomized controlled efficacy trial with the primary objective to assess whether consumption of biofortified yellow cassava can improve the serum retinol concentration of marginally deficient children. A sensory and cultural acceptability study to assess which factors influence mothers to prepare yellow cassava for their children is described in chapter 3. Chapter 4 describes a study that was designed to assess the prevalence of vitamin A deficiency among children in 15 primary schools. We also used this study to validate new methods for measuring vitamin A deficiency. Dietary intake data from the children in the efficacy trial was used to study whether provision of yellow cassava could fulfill their recommended nutrient intake levels using linear programming (chapter 5). Finally, chapter 6 discusses the main findings and conclusions of this thesis and puts these in a public health perspective including recommendations for possible future research.

Study site selection

We selected the study area based on the following criteria: expected presence of vitamin A deficiency in school children; high number of primary schools with more than 350 eligible children; nearby fields for cassava production; and no other projects ongoing in the area that could possibly interfere with the yellow cassava study. Based on the selection criteria described above, we conducted our research around Kibwezi district, Makueni County in Eastern province of Kenya (Figure 4). This rural semi-arid area is inhabited predominantly by the Kamba community. There are two rain-fall seasons, the short rains in November and the long rains in April-May but these are often erratic and unreliable. The community consists mostly of agro-pastoralists engaged in rain fed agriculture and the main crops planted are maize, green grams and pigeon peas. Although cassava is not unknown in the area, it is not a major crop.

The research was carried out within the INSTAPA project (www.instapa.org); an EU/FP7-funded multi sectorial project on novel staple food-based strategies to improve micronutrient status for better health and development of women and children in sub-Saharan Africa. The project is conducted by research scientists from Europe, Africa and USA and focuses on the improvement of millet-, sorghum-, maize-, and cassava-based foods in sub-Saharan Africa to safely prevent deficiencies of iron, zinc, and vitamin A and to improve immune function and cognitive development.



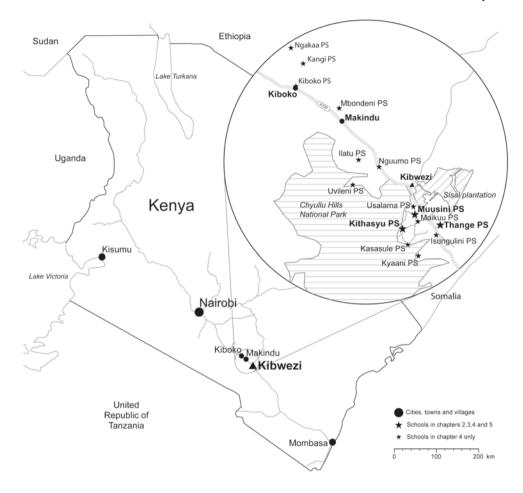


Figure 4: Map of Kenya with Makindu and Kibwezi district and primary schools in the described research

(see annex for detailed map of the households)

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Abstract

Background: Biofortified yellow cassava is naturally rich in β -carotene and holds potential to alleviate vitamin A deficiency. We assessed the effect of consuming yellow cassava on serum retinol concentration in Kenyan schoolchildren with marginal vitamin A status.

Methods: We randomly allocated 342 children aged 5–13 years to receive: 1) white cassava and placebo supplement (Group 1); 2) yellow cassava (containing ~1,460 μg β-carotene) and placebo (Group 2), white cassava and β-carotene (1,053 μg) supplement (Group 3); 6 days/week for 18.5 weeks. The primary outcome was serum retinol concentration; secondary outcomes were serum concentrations of β-carotene, retinol-binding protein, transthyretin; and hemoglobin concentration. Groups were compared using ANCOVA, adjusting for inflammation, baseline values and for the stratified randomization. This study is registered with ClinicalTrials.gov (NCT01614483).

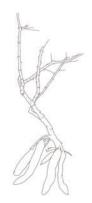
Results: The study was conducted between May and November 2012. At baseline, 27% of children were vitamin A deficient and 24% had inflammation. Among children included in the per protocol analysis (n=335), compliance to cassava feeding was 100%. We daily supplied groups 1, 2 and 3 the equivalent of 22 μ g, 220 μ g and 175 μ g retinol, respectively. Both yellow cassava and β -carotene supplementation increased serum retinol concentration by 0.04 μ mol/L (95%CI: 0.00–0.07 μ mol/L); corresponding effects on serum β -carotene concentration were 524% (448%–608%) and 166% (134%–202%). In pre-planned subgroup analyses, we found no evidence of effect modification by initial vitamin A status, zinc status, or polymorphisms in the β -carotene monooxygenase gene.

Conclusions: Consumption of yellow cassava is a promising and new approach to control vitamin A deficiency.

Chapter 2

Biofortified yellow cassava and vitamin A status in Kenyan children: a randomized controlled trial

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Introduction

Biofortified yellow cassava has great potential to alleviate vitamin A deficiency, complementary to other interventions such as vitamin A supplementation and fortification^[1,2]. Vitamin A deficiency is widely prevalent in sub-Saharan Africa despite national supplementation programs. In Kenya, for instance, 60% of preschool children are deficient^[3]. Because vitamin A supplementation in preschool children reduces all-cause mortality by $24\%^{[4]}$ and considering the extent of vitamin A deficiency, even small increases in the supply of vitamin A through biofortified crops are likely to result in major public health gains.

Cassava is an important staple food for many people in developing countries, particularly in Africa but also in large parts of Latin America and Asia. The crop is well suited to arid and semi-arid areas, and is grown even in remote areas by poor subsistence-farming families with the highest burden of vitamin A deficiency. Children from such families are difficult to reach through supplementation or fortification. Because cassava is multiplied through vegetative propagation, farmers can grow improved varieties indefinitely with marginal inputs^[5]. Cassava varieties grown in Africa have white roots with virtually no provitamin A. There are clones originating in the Amazon Basin with yellow roots due to the natural presence of provitamin A. These yellow varieties have recently been cross-bred using conventional techniques with African cassava varieties to increase provitamin A contents^[6].

Yellow cassava contains provitamin A carotenoids primarily as β -carotene, which must be absorbed and converted to retinol (vitamin A). Because of differences in the food matrix, this bioefficacy varies greatly between crops but is generally higher for roots and tubers than for dark-green leafy vegetables^[7]. In addition, conversion of β -carotene may depend on mutations in the gene encoding for 15,15- β -carotene monooxygenase (BCMO1), the enzyme that converts provitamin A carotenoids to retinol^[8,9]. This conversion is thought to depend on vitamin A status, and to be more efficient in deficiency^[10,11]. Zinc deficiency can impair provitamin A bioefficacy because it is essential for transport and metabolism of vitamin A^[12].

We aimed to assess the effect of consuming biofortified, yellow cassava on serum retinol concentration in Kenyan children with mild to moderate vitamin A deficiency. In pre-planned subgroup analyses, we also explored effect modification by BCMO1 genotype and for vitamin A and zinc status.

Materials and methods

Study design

The study was designed as a randomized controlled trial with three parallel arms with controlled intake of:

- 1. White cassava and placebo supplement ('Control group')
- 2. Provitamin A-rich cassava and placebo supplement ('Yellow cassava group')
- 3. White cassava and β -carotene supplement (' β -carotene supplement group').

The latter group was included as a positive control.

The study was registered (clinicaltrials.gov: NCT01614483), approved by ethical committees in Kenya and the Netherlands, with oversight by a data safety monitoring board. Consent was obtained from parents and children.

Subjects and screening

The study was conducted from May until November 2012 in three primary schools in Kibwezi District, Kenya. We selected all children aged 5-13 years, and collected capillary blood samples in a tube with EDTA. Throughout the study, blood samples were obtained from fasted children. We assessed plasma concentrations of retinol-binding protein (see below) as a proxy for vitamin A status, hemoglobin concentration (Hemocue 201+, Ängelholm, Sweden), and C-reactive protein concentration by point-of-care test (QuikRead, Orion Diagnostica, Espoo, Finland). We also assessed Plasmodium infection using dipstick tests specific for P. falciparum histidine-rich protein-2, and lactate dehydrogenase specific to either P. falciparum or non-falciparum human Plasmodium species (CareStart G0121 and G0171; AccessBio Inc., Sommerset, NJ). Children with inflammation (C-reactive protein concentration > 8 mg/L) or Plasmodium infection were excluded; of those remaining, we selected 360 children with the lowest retinol binding protein concentrations. At the start of a 2-week run-in period, these children received praziquantel (40 mg/kg bodyweight) and albendazole (100 mg as a single dose) to prevent helminth infections. During the run-in period, they were daily offered servings of boiled white cassava and capsules with placebo supplements. At the end of the run-in period ('baseline'), children were excluded who had missed >20% of the feeding sessions or who were unable to consume ≥80% of their portion size of 325 g (5-8 years) and 375 g (9-13 years).

At the end of the run-in period, we collected venous blood from each child in a tube without anticoagulant and suitable for trace element analysis (Becton Dickinson, Franklin Lakes, NJ), and in a tube with K2EDTA (Becton Dickinson, Franklin Lakes, NJ). Weight and height were measured according to WHO guidelines $^{[13]}$ to the nearest 0.1 kg and 0.1 cm using a mechanical floor scale and a portable stadiometer (Seca, Hamburg, Germany). We excluded children with inflammation (C-reactive protein concentration > 8 mg/L), Plasmodium infection or a 14-day history of infectious or systemic disease.

Randomization and blinding

Children were allocated by stratified block randomization, after baseline data collection. Randomization was done by one of the authors (HV) not involved in the field work, based on a list with child names and corresponding plasma retinol-binding protein concentrations measured before the run-in period. Tables with random numbers were used to generate the allocation sequence consisting of random permuted blocks with size 6 or 9 within each of three strata. This sequence was used to allocate children to one of the three possible intervention groups, with stratum corresponding to tertiles of retinol-binding protein concentration. The allocation list contained each child's name, and a group code letter A-C. Both the field team and participants were unblinded to the type of cassava (white or yellow), but were blinded to supplementation with β -carotene or placebo. Supplements were formulated as opaque capsules to allow the field team and study participants to remain blinded to their contents.



Interventions and follow-up

White cassava and seven different varieties of yellow cassava were grown at a location close to the study site, with stacked planting to allow roots to be harvested at 7-10 months after planting. Yellow varieties had been screened and pre-selected based on low cyanide concentration and suitability for freshly boiled consumption. Cyanide content was re-checked for each variety before consumption using the method described by Fukuda et al[14]. Cassava was harvested daily in the afternoon and prepared the subsequent morning. At each school, roots were peeled, chopped, rinsed twice and boiled for one hour or until done in separate pots for white and yellow cassava. Cassava was drained, mashed with oil and salt as per standardized recipe and served warm as a midmorning snack, 6 days per week for 18.5 weeks. During feeding sessions, intervention groups were physically separated at each school and monitored to avoid food sharing and spilling. Target portions were >325 g and >375 g for children aged 5-8 years and 9-13 years, respectively. The amount of cassava eaten was recorded daily for each child as the difference in weight of the serving after and before eating. At each school, interventions started for all children simultaneously, 2 days after the last day of the run-in period.

Supplemental capsules contained either 1,053 μ g β -carotene (Betatap 20% S, DSM Nutritional products, Switzerland) or no active ingredient (placebo), with starch as filler. Capsules were administered directly after feeding and were swallowed with water under supervision. In addition to the experimental cassava, we provided daily voluntary lunches with cooked maize and beans.

Portions (375 g) of cooked white and yellow cassava were sampled daily at each school and mixed with a food processor after adding the preservative antioxidant tert-butylhydroquinone (2.5 mL/kg) solved in methanol (20 g/100 mL). Duplicate samples (15 g/day) were pooled by week per school and stored at -15° C in Kenya, transported and stored at -80° C in the Netherlands.

Upon completion of the intervention period, we repeated blood and data collection using the same procedures as at baseline, except that the trace element tube was replaced by a serum separation tube (Becton Dickinson). Whole blood was stored in DNA-stabilizing buffer (AS1, Qiagen, Valencia, CA) and kept at 4°C during transport to the Netherlands until DNA analysis. Serum samples were shielded from light, processed under subdued light conditions and kept in amber cryovials at -196°C in the field and -80°C during transport and storage until assessment of retinol concentrations in the Netherlands. For ethical reasons, all children received supplements with vitamin A (100.000IU) at the end of the study.

24 hour recall data

In week 13-16 after randomization, mean daily intake of energy, fat and vitamin A were measured in 334 subjects by quantitative 24-hour recall^[15] and repeated in a subsample (n=101) on non-consecutive days. Trained interviewers asked caretakers to name all food and drinks consumed by their child during the preceding day, and to describe ingredients and cooking methods of mixed dishes. Duplicate amounts of all foods, beverages, and ingredients of mixed dishes consumed were weighed to the nearest 2 g (Soehnle electronic scale, model 65086; Plateau Art, Germany) or, when not available, amounts were estimated either in household units, volume, or monetary value. The proportion consumed was estimated from total volume of food cooked at the respondents' household and the food volume

consumed by the child; this proportion was multiplied by the total amount of ingredients used in preparing the dish to determine the amount of ingredients consumed. Standard recipes were generated to account for foods consumed outside the home. Conversion factors were determined from household units, volume and monetary values to weight equivalent of each ingredient. Nutrient intake was calculated based on a food composition table developed specifically for the study, using the national food composition table of Kenya^[16] as primary source, complemented with data from food composition tables from South Africa^[17], Mali^[18], the International Minilist (IML)[19] and the US Department of Agriculture[20]. USDA retention factors release 6[21] were applied to raw ingredients and foods to account for nutrient losses during food preparation. Contents of vitamin A, β-carotene and retinol were converted into retinol activity equivalents (RAEs) with conversion factors as per international recommendations^[22]. Conversion factors for β -carotene from yellow cassava and supplements were conservatively assumed to be 7:1. Intakes of energy, fat and vitamin A were adjusted for day-to-day variation^[23,24] to estimate usual intakes for each child.

Biochemical analyses

Plasma retinol-binding protein concentration was measured in duplicate by two-site enzyme linked immunoassay (K-assay KT-504, Kamiya Biomedical Company, Seattle, US) with an iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, USA; intra-assay CV: 2.4%, inter-plate CV: 1.5% in 32 plates). At baseline and at end of study, we measured hemoglobin concentration within 6 hours after blood collection using a hematology analyzer (Celltac-a, MEK-6410K, Nihon Kohden Corporation, Tokyo, Japan).

Serum concentrations of retinol and β-carotene

Serum collected at baseline and end of intervention were analyzed (May 2013) in pairs to reduce analytical variation. Concentrations of retinol and other carotenoids were measured by HPLC (Thermo Scientific Accela LC system, Thermo Fisher Scientific, Waltham, MA, USA) and analyzed using EZChrom Elite version 3.2.2 SP2 software (Agilent Technologies, Santa Clara, CA). For this, 500 µL of serum, 500 µL NaCl (0.9 w/v% in water) and 1,000 µL ethanol (with added retinyl acetate as internal standard) were mixed and extracted twice with 30 mL hexane. The hexane layers were pooled and evaporated to dryness in a vacuum concentrator at 35°C (RVC 2.25 CD plus, MartinChrist, Osterode-am-Harz, Germany). The residue was dissolved in a 250 µL mixture of methanol and butanol (60/40 v/v %); 15 μL was injected per HPLC analysis. Sample preparations were done under subdued yellow light. Retinol, retinyl acetate, and lutein, zeaxanthin, β-cryptoxanthin, a-carotene, β-carotene and lycopene were separated on a Vydac 201TP52 RP-column using gradient elution and monitored at 325 nm (retinol and retinyl acetate) and 450 nm (carotenoids) on a photodiode array detector. Runtime was 25 min per sample. Within- and between-run CVs for high and low controls were 3.1% and 2.7% for retinol (level \sim 60 μ g/100 mL), and 9.4% and 11.1% for β -carotene (level ~10 μ g/100 mL).



Cassava B-carotene concentration

Carotenoids in cassava were analyzed using the same HPLC system as described above for serum samples. To extract carotenoids from cassava, we mixed 2 g homogenized cassava, 0.2 g magnesium carbonate, 5 mL deionized water and 1000 μ L ethanol (with added retinyl acetate as internal standard), and extracted

three times with 20 mL methanol-tetrahydrofuran (1:1 v/v%) using a rod mixer (Polytron PT 20 OD, Kriens/Luzern, Switzerland) until the residue was colorless. Extracts were filtered on a glass funnel with Whatman paper; the combined filtrates were transferred to a 50 mL volumetric flask and made up to volume with methanol-tetrahydrofuran (1:1 v/v%). 4 mL filtrate and 1 mL 10% NaCl-solution was transferred to a 10 mL Kimax tube and carotenoids were extracted three times with 1.5 mL petroleum-ether containing 0.01% butylated hydroxytoluene. The combined ether fractions were evaporated under nitrogen at 35°C. The residue was dissolved in 2 mL methanol/butanol (60/40 v/v%) and 1 μ L was injected into the HPLC system. Carotenoids were separated on a Vydac 201TP52 column using gradient elution and monitored at 450 nm on a photodiode array detector. Runtime was 20 min per sample. Within- and between-run CVs for high and low control for β -carotene were 3.9% and 7.7% (level ~470 μ q/100 q).

Serum retinol-binding protein concentration

For serum samples collected at the end of intervention, retinol-binding protein concentrations were determined by ELISA (Quantikine DRB400, R&D Systems, Minneapolis, MN). Results were read in duplicate for 10% of samples. Inter-plate CV for five plates was 5.4% and intra-assay CV was 2.9%.

Iron and inflammation markers in serum

Iron markers (concentrations of ferritin, soluble transferrin receptor) and inflammation markers (concentrations of C-reactive protein and a1-acid glycoprotein), transthyretin concentration and zinc concentration were measured at the Meander Medical Hospital, Amersfoort, the Netherlands, on a Beckman Coulter UniCel DxC 880i analyzer as per manufacturer's instructions.

SNPs

DNA was isolated (FADWE002, Favorgen Biotech Corporation Ping Tung, Taiwan) and SNPs in the BCMO1 gene were scored from 0 to 1 using the Assay Design Tool (Illumina Technical Support) based on compatibility to successful GoldenGate genotyping. SNPs with a score >0.4 (rs12934922, rs11645428, rs7501331, rs6564851 and rs6420424) were genotyped by Illumina's VeraCode™ GoldenGate Genotyping Assay on a BeadXpress™platform using the VeraCode™ technology. SNP clustering was also assessed visually to determine success of genotyping. SNPs with a GenCall score >0.5 and a call rate ≥0.90 were included in the final analysis. Of 5 SNPs analyzed, one (rs12934922) yielded no detectable signal and was excluded for analysis. For several children, DNA qualities were low, resulting in 56–88 children being excluded depending on SNP-type.

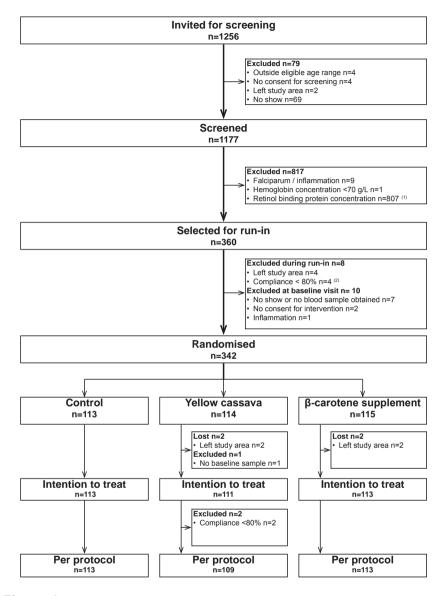


Figure 1: Flow of participants

- ¹ The upper segment of retinol binding protein concentrations were not eligible for run-in and excluded
- ² Children who could not consume 80% of their respective portion size were excluded



Statistical analyses

Anthropometric indices were calculated by ANTHRO-plus (WHOv3.2.2, www.who. int/childgrowth/software/en/). Data were analyzed using IBM-SPSS (version 21) and CIA (version v2.2.0 b57; http://www.som.soton.ac.uk/cia/). Vitamin A deficiency was defined by serum retinol concentration <0.7 μ mol/L^[25]. Zinc deficiency was defined by serum zinc concentration <9.9 μ mol/L^[26]. Anemia was defined by hemoglobin concentration <115 g/L for children aged 5–11 years and <120 g/L for children aged 12–13 years^[27]. Iron deficiency was defined by serum ferritin concentrations <15 μ g/L^[27] and serum soluble transferrin receptor concentration >21 nmol/L (1.55 mg/L)^[28]. Inflammation was defined by either serum concentrations of C-reactive protein >5 mg/L or *a*1-glycoprotein protein concentration >1 g/L^[29].

Compliance to treatment was defined by the total amount of cassava consumed during the intervention as a fraction of the age-specific target amount to be consumed for the total duration of the intervention.

Our pre-planned primary endpoint was serum retinol concentration at end of intervention; secondary endpoints were serum concentrations of β-carotene, retinol binding protein and transthyretin, and hemoglobin concentrations. Distributions of dependent variables were checked for normality and log-transformed for serum β-carotene concentration. The primary analysis was per protocol and restricted to children who attended >80% of intervention days and consumed >80% of the target amount of cassava over the total intervention period. For primary and secondary analyses, we used ANCOVA to compare intervention groups, with adjustment for the stratified design; serum concentrations at baseline of C-reactive protein, a1-acid-glycoprotein, retinol, and zinc; gender; and anemia, with a backward elimination procedure. Analyses of secondary outcomes were adjusted accordingly. We assessed effect modification of baseline vitamin A and zinc status by stratified analyses and by multivariate regression analysis. In the analyses of effect modification by SNP genotype, we combined the groups receiving yellow cassava and β-carotene supplements to increase the sample size for each genotype.

Results

Of 1,256 screened children, 342 (27%) were randomized. Exclusions were mostly because we selected for children with low plasma concentrations of retinol-binding protein. Four children were lost to follow up, one child did not have a baseline sample and two children did not meet compliance criteria, resulting in 335 children in the per protocol analyses (Figure 1).

At baseline, mean serum retinol concentrations were 0.80 μ mol/L, 0.83 μ mol/L and 0.82 μ mol/L in the groups receiving control, yellow cassava and β -carotene supplements, respectively (Table 1), indicating a study population with marginal vitamin A deficiency. One-quarter of children had inflammation as indicated by elevated concentrations of C-reactive protein, a1-acid glycoprotein or both. When these children were excluded, the prevalence of vitamin A deficiency was 24%, 24% and 23% in the groups receiving control, yellow cassava and β -carotene supplements, respectively. The corresponding prevalence values for zinc deficiency were 5%, 1% and 4%; for anemia 7%, 7% and 6%; and for iron deficiency 31%, 39% and 42%.

Table 1. Baseline characteristics, by intervention group (per protocol analysis)

	Control	Yellow cassava	β-carotene supple ment
n	113	109	113
Vital and personal characteristics			
Age, years	8.9 (2.1)	8.8 (2.4)	8.9 (2.4)
Sex, girls	52 (46%)	55 (50%)	58 (51%)
Body-mass-index-for-age z-score, SD	-1.4 (0.9)	-1.5 (0.9)	-1.5 (0.9)
Height-for-age z-score, SD	-1.2 (1.2)	-1.1 (1.1)	-1.3 (1.0)
Children being stunted	24 (21%)	22 (20%)	22 (20%)
Inflammation markers			
Serum C-reactive protein concentration, mg/L	0 [0,0]	0 [0,0]	0 [0,0]
Serum a1-glycoprotein protein concentration, g/L	0.87 (0.26)	0.87 (0.23)	0.86 (0.20)
Serum C-reactive protein concentration >5 mg/L	3 (2.7%)	4 (3.7%)	1 (0.9%)
Serum concentration C-reactive protein >5 mg/L or a1-glycoprotein >1 g/L	29 (26%)	25 (23%)	26 (21%)
Vitamin A markers, all children			
Serum retinol concentration, µmol/L	0.80 (0.17)	0.83 (0.17)	0.82 (0.18)
Serum transthyretin concentration, g/L	0.18 (0.03)	0.19 (0.03)	0.18 (0.03)
Serum β-carotene concentration, μmol/L	0.34 [0.24-0.48]	0.35 [0.24-0.56]	0.35 [0.24-0.48]
Vitamin A deficiency 1	31 (27%)	29 (27%)	30 (27%)
Vitamin A status markers, children without inflammation	n²		
Serum retinol concentration, µmol/L	0.82 (0.17)	0.84 (0.17)	0.83 (0.17)
Vitamin A deficiency ¹	20 (24%)	20 (24%)	20 (23%)
Zinc markers, all children			
Serum zinc concentration, µmol/L	13.5 (2.3)	14.3 (2.0)	14.0 (2.3)
Zinc deficiency ³	4 (4%)	1 (1%)	3 (3%)
Zinc markers, children without inflammation ²			
Serum zinc concentration, µmol/L	13.1 (2.3)	14.2 (1.8)	14.0 (2.2)
Zinc deficiency ³	4 (5%)	1 (1%)	3 (4%)
ron markers, all children			
Haemoglobin concentration, g/L	131 (10)	130 (12)	132 (12)
Serum ferritin concentration, µg/L	18.4 [11.4-24.2]	17.8 [11.2-25.6]	15.6 [10.4-24.4]
Serum soluble transferrin receptor concentration, mg/L	1.72 [1.51-2.05]	1.83 [1.60-2.12]	1.86 [1.62-2.17]
Anaemia ⁴	8 (7%)	8 (7%)	7 (6%)
Iron deficiency 5	33 (29%)	44 (40%)	48 (43%)
Iron deficiency anaemia ⁷	6 (5%)	7 (6%)	5 (4%)
Iron markers, without inflammation ²			
Serum ferritin concentration, µg/L	17.1 [10.8-22.3]	17.5 [11.4-24.5]	15.4 [10.1-23.0]
Iron deficiency 5	26 (31%)	33 (39%)	37 (42%)
Iron deficiency anaemia ⁶	5 (6%)	4 (5%)	4 (5%)



 $^{^{1}~}$ Vitamin A deficiency: serum retinol concentration < 0.70 $\mu mol/L$

 $^{^2}$ Defined by serum concentrations of C-reactive protein > 5 mg/L and/or $\it a1$ -acid glycoprotein > 1 $\mu g/L$

 $^{^3}$ Zinc deficiency: serum zinc concentration < 9.9 μ mol/L

 $^{^4}$ Anaemia: haemoglobin concentration < 115 or <120 g/L for children aged 5–11 years and > 12 years, respectively

 $^{^{5}}$ Iron deficiency: serum ferritin concentration < 15 μ g/L and soluble transferrin receptor concentration > 1.55

⁶ Iron deficiency anaemia: iron deficiency with concurrent anaemia.

Compliance to the cassava feeding was 100% with an average portion size of 336 gram and 382 gram for children aged 5–8 years and 9–13 years, respectively (Table 2). The average daily intake from the general diet was ~20 μ g retinol-activity equivalents (RAE); fat contributed only to 14% of total energy intake. The RAE intakes were 22 μ g, 220 μ g and 175 μ g in the groups receiving control, yellow cassava and β -carotene supplements. We found no Plasmodium infection at the end of intervention.

Table 2. Daily intake of energy, fat, vitamin A and β -carotene from diet and interventions

	Control	Yellow cassava	β-carotene supplement
n	113	109	113
General diet (excluding experimental cassava) ¹			
Energy intake from, kJ	5,739 [4,175-8,049]	5,673 [4,605-6,988]	5,863 [4,391-7,929]
Fat, % energy	13.1 [11.3-16.6]	14.1 [12.3-16.9]	14.0 [12.0-15.9]
Vitamin A intake, μg RAE ²	16.9 [4.6-38.6]	13.8 [4.0-46.1]	21.1 [9.5-40.5]
Children without any preformed retinol	86 (77%)	83 (76%)	74 (65%)
Supplied by intervention			
Amount of cassava eaten daily, g ³	378 [339-383]	371 [333-381]	378 [340-384]
β-carotene from cassava, μg	38 [34-39]	1,463 [1,313-1,501]	38 [34–39]
β-carotene from supplement, μg	0	0	1,053 [1,044-1,053]
Vitamin A supplied by general diet and intervention, $\mu g \ \text{RAE}^{\scriptscriptstyle 4.5}$	22 [10, 44]	220 [205-241]	175 [164-195]

Values indicate median [25th- 75th percentiles] or n (%) unless indicated otherwise

In the primary analysis, both yellow cassava and supplementation with β -carotene increased serum retinol concentration by 0.04 μ mol/L (95%CI: 0.00, 0.07 μ mol/L) (Table 3, Figure 2). Intention to treat analyses showed similar effects (not shown).

The corresponding effects on serum β -carotene concentration were 524% (448%, 608%) and 166% (134%, 202%), respectively. Interventions did not change the prevalence of vitamin A deficiency, serum concentration of retinol-binding protein, transthyretin, or hemoglobin concentration.

There was no evidence that intervention effects varied by initial vitamin A status or initial zinc status (Figure 3A), or BCM01 genotype (Figure 3B). For rs7501331, all children except for one were wild type. Minor allele frequencies were 0.09 for rs11645428, 0.60 for rs6564851 and 0.56 for rs6420424 and all SNPs except rs7501331 were in Hardy–Weinberg equilibrium.

 $^{^{1}}$ Based on 24 hour recall data of n=111 in control, n=113 in β -carotene supplement and n=108 in yellow cassava;

 $^{^2}$ Bioconversion mix diet: 12 μg β -carotene is absorbed and converted to 1 μg retinol, (IOM, 2001)

³ Cassava was cooked in water for 1 hour, any leftover water discarded, and mashed with 12 g oil and 8 g of salt per kg cooked cassava

 $^{^4}$ Based on average β-carotene concentration of 3.94 (0.75) μ g/g (fresh weight) and 0.10 (0.03) μ g/g for boiled yellow and white cassava respectively

 $^{^{5}}$ Assumed bioconversion from supplement and cassava: 7 μg β -carotene to 1 μg retinol

Table 3. Effects of consumption of yellow cassava and supplementation with β-carotene on various outcomes

	0	Control	Yel	Yellow Cas-	Effect (95% CI)	15% CI)	B-car	β-carotene sup-	Effect (95% CI)	5% CI)
				sava	•		pler	plementation	•	
	ב	Estimate	_	Estimate	Crude	Adjusted	ב	Estimate	Crude	Adjusted
Serum retinol concentration, µmol/L	113	0.77 (0.01)	109	0.81	0.05 (0.01,0.10)	0.04 (0.00,0.07)	113	0.81	0.05 (0.00,0.09)	0.04 (0.00,0.07)
Prevalence of vitamin A deficiency (retinol concentration <0.7 μmol/L)	113	30%	109	27%	3.5% (-8.3%,15.1%)	AN	113	27%	0.9% (-10.9%,12.7%)	AN
Serum β-carotene concentration, μmol/L	113	0.141	109	0.861	537% (453%,633%)	524% (448%,608%)	112	0.371	164% (130%,203%)	166% (134%, 202%)
Serum retinol-binding protein concentration, µmol/L	112	0.62 (0.01)	108	0.62 (0.01)	0 (-0.04,0.04)	0 (-0.04,0.04)	113	0.65 (0.01)	0.03 (-0.01,0.07)	0.03 (-0.01, 0.01)
Serum transthyretin concentration, g/L	113	0.18	108	0.18	0 (-0.01,0.01)	0 (-0.01,0.01)	111	0.18	0 (-0.01,0.01)	0 (-0.01,0.01)
Haemoglobin concentration, g/L	112	130.6 (0.7)	109	130.4 (0.7)	-0.32 (-3.12, 2.48)	-0.17 (-2.11,1.77)	112	129.9 (0.7)	-0.04 (-2.82,2.74)	-0.69 (-2.62,1.24)

NA: not applicable.

Estimates are given for adjusted analysis only.

'Crude' effects were adjusted for stratified design only. In the analysis of each outcome, we also adjusted for the same marker measured at baseline (e.g. in the analysis of serum retinol concentration, we adjusted for serum retinol concentration chaseline not measured). In the analysis of serum retinol concentration and serum retinol binding protein concentration and serum retinol binding protein concentration, we also adjusted for serum concentrations of C-reactive protein and \(\alpha 1-\text{glycoprotein}\) measured at the end of intervention. Serum \(\text{p-cancer}\) Values indicate arithmetic means (SE), 'geometric means or prevalence.

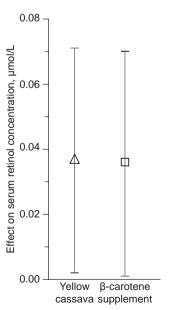


Discussion

Overall, daily consumption of yellow cassava resulted in a modest increase in serum retinol concentration and a large increase in β -carotene concentration. There was no evidence that the effect on serum retinol concentration depended on initial vitamin A status, zinc status or BCM01-genotype.

This is the first randomized intervention with yellow cassava conducted in a target population where vitamin A deficiency is common. The dropout rate in our study was exceptionally low with only four children lost to follow up. Also the compliance to the feeding was very high with almost all children consuming more than their assigned portions sizes and only two children failing to finish their rations on a daily basis. Therefore, inferences from this study are not limited by shortcomings in study conduct.

Although the intervention effect of 0.04 μ mol/L on serum retinol concentration in the yellow cassava group was modest, it shows that β -carotene from yellow cassava was converted to retinol. Strikingly, serum retinol concentration dropped in the control group whereas in the yellow cassava and β -carotene supplement group the concentration did not change. This may have been due to a seasonal change in availability of foods rich in β -carotene^[30]. In contrast to the modest increase in serum retinol, the serum β -carotene concentration increased remarkably, showing that β -carotene from yellow cassava was well absorbed. This indicates that certain factors may have hampered the bioconversion of β -carotene to retinol.



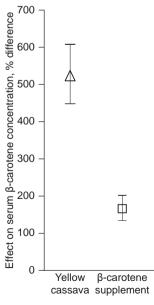


Figure 2: Intervention effect on serum concentrations of retinol (left panel in µmol/L) and B-carotene (right panel in % difference) In the analysis of serum retinol concentration we adjusted for the stratified design, serum retinol concentration at baseline and concentrations of serum C-reactive protein, a1-acid-glycoprotein concentration at the end of intervention. In the analysis of serum β-carotene we adjusted for the stratified design, and serum B-carotene concentration at baseline.

Some food based trials with provitamin A rich vegetables and fruits have previously shown larger effects on serum retinol concentration and smaller effects on β -carotene concentrations^[31,32] as compared to our study. In a study among South African children, consumption of biofortified sweet potato did not change serum retinol concentration but vitamin A pool did increase slightly, despite providing much more β -carotene than yellow cassava (12,375 μ g versus 1,463 μ g from yellow cassava) and despite a vitamin A deficiency prevalence of ~70%^[33]. Also, in a study in Bangladeshi women, daily consumption of biofortified sweet potato resulted in a large increase in serum β -carotene concentration, but had no evident effect on serum retinol concentration^[34].

Many factors may have an effect on the bioconversion of β -carotene and these factors can be either food or host related^[35]. The Institute of Medicine recommends a conversion factor of 12:1 for β -carotene from mixed diets^[23], but a study with yellow cassava in gerbils showed a bioconversion factor of 3.7: 1^[36]. Another study with yellow cassava in 10 American women showed a conversion factor of 4.2: 1^[37], although inter-individual variation was high (range 0.3 to 10.6). Based on these studies, it seems unlikely that food related factors play a significant role in the bioconversion of β -carotene from yellow cassava.

Host related factors that may affect bioconversion of β -carotene have not been studied as extensively as food-related factors. We expected to find a larger intervention effect in vitamin A deficient children. Absence of this effect in the subgroup analyses could be explained by the children only being marginally deficient, whereas a modifying effect is expected to be more pronounced in severely deficient children^[10]. Zinc deficiency has also been suggested to reduce enzymatic cleavage of β -carotene^[38]. However, we only found four children to be zinc deficient and the effect in our study is therefore negligible.

Poor conversion might be explained by mutations in the gene that encodes for B-carotene 15,15'-monooxygenase (BCMO1), the key enzyme in enterocytes that cleaves β-carotene into two molecules of retinal, a product that is subsequently reduced to retinol^[8,39]. In their favor, our study subjects were nearly all homozygous wild-type for rs7501331 (A379V) which is in agreement with the limited data on African populations in the HapMap database^[40]; however, it is commonly found in Caucasian populations and was reported to reduce β-carotene conversion by 32%^[9]. None of the other SNPs that we assessed showed significant effect modification, although rs11645428 wild type subjects tended to have a lower response to treatment as compared to heterozygous subjects, which would be in agreement with the 51% reduction in β-carotene conversion reported by Lietz et al.[8]. Rs11645428 has a risk allele frequency of 0.66 in Caucasians, whereas this was 0.91 in our population. We encountered no subjects homozygous for the minor allele and therefore could not make any further inferences for this SNP. Whether this or yet unidentified SNPs negatively affect β-carotene conversion in African populations as compared to Caucasian populations needs to be further elucidated. For now, we can conclude that these SNPs in the BCMO1 gene did not clearly reduce the effect of yellow cassava on retinol concentration in our study.



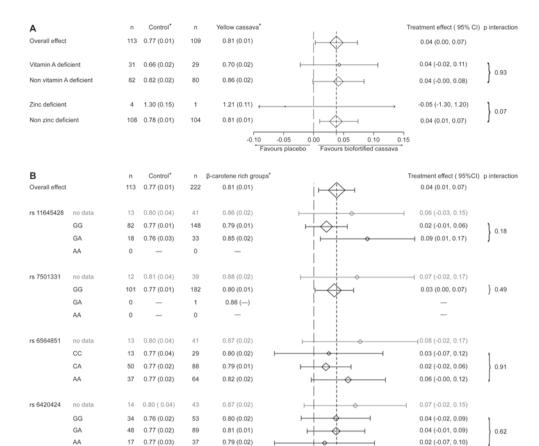


Figure 3: Effect of yellow cassava (panel A) and increased intake of β-carotene (panel B) on serum retinol concentration (μ mol/L), by subgroups

0 -0.05 Favours placebo

Favours β-carotene

* adjusted means (SE). Genotypes on top are wildtype. In the analyses of serum retinol concentration we adjusted for the stratified design, serum retinol concentration at baseline and concentrations of serum C-reactive protein and a1-acid-glycoprotein concentration at the end of intervention To maximize the supply, absorption and conversion of β -carotene, we controlled for several factors in this study. First, cassava was prepared by boiling, having the highest retention of β -carotene compared to other preparation methods^[41]. Second, cassava intake was strictly controlled, in substantial amounts (median: 375 g/child/day) and sustained for more than four months. Third, we included marginally deficient children of which one-quarter was deficient, since effectiveness of dietary interventions with β -carotene may depend on vitamin A status^[10,11]. Fourth, we dewormed the children twice and added oil to the boiled cassava to increase absorption of the β -carotene in the small intestine. As a measure of intake, the increase in serum β -carotene concentrations showed that this strategy was indeed successful.

A limitation in the measurement of vitamin A status is that although serum retinol concentration is the reference method for measuring vitamin A status $^{[3,42]}$, it is under homeostatic control of the liver and does not reflect hepatic vitamin A stores anymore at high concentrations $^{[43]}$. This effect is expected to be seen at a serum retinol concentration of >1.05 μ mol/L, whereas the children in our study were clearly in the mild deficient range (~0.80 μ mol/L) and serum retinol concentration would be expected to be linearly associated with hepatic stores $^{[43]}$. Isotopic dilution methods can be used to estimate body retinol pool size $^{[44,45]}$ and provide an alternative to serum retinol, but require more sophisticated equipment and were therefore not used.

The question arises whether an increase in serum β -carotene concentration in these and similar studies has functional significance. Circulating β -carotene is taken up and concentrated in various tissues [46]. Thus liver and adipose tissue are major storage sites for β -carotene and other provitamin A carotenoids, whilst kidney and lung tissues have minor roles for storage [47]. In steady state, only 1% of the total body content of carotenoids is contained in serum [48]. Recent studies have shown that BCMO1 is not only expressed in enterocytes, but also in various cell types in a broad variety of human tissues including liver tissue [39,49]. The expression and activity of BCMO1 in extra-intestinal tissues indicate that these tissues have the capacity to meet their vitamin A needs directly by converting circulating or locally stored carotenoids [39,49]. The hypothesis that the most important conversion into vitamin A takes place in selected tissues and not only in the intestine [47] would explain the efficacy of orally dosed β -carotene in reducing manifestations of vitamin A deficiency as shown in several trials [50,51,52].

Our study was conducted in primary school children and not in pre-primary school children who are more at risk of vitamin A deficiency $^{[3,25]}$. Older children were chosen because; first, to reduce possible interference with the national vitamin A supplementation program for children under 5 years of age; second, to establish higher cassava and β -carotene intake as older children are able to consume more food; and third, for logistical reasons as the primary school setting provided us with a higher level of control than home feeding and allowed us to feed a large group of children together. However, our results can be extrapolated to younger children with the notion that we assume that yellow cassava will have a larger impact in the younger age groups since they are more likely to be vitamin A deficient especially in areas where vitamin A supplementation programs are poorly implemented.

We used seven different yellow varieties with a β -carotene concentration range of 4.7 to 6.9 μ g/g (fresh weight), which is still below the breeding target levels



of 15 μ g/g for cassava^[53]. Furthermore, cassava breeding is an ongoing, rapidly progressing process and experimental varieties are currently available with total provitamin A content of 22 μ g/g (personal communication, H Bouis 2014). These new varieties can therefore be expected to have a larger impact on vitamin A status than we have shown here. Acceptance of the crop does not seem to pose any difficulties as we conducted a consumer acceptability study in our research area and found that yellow cassava was liked even more than white cassava^[54].

Biofortification is a promising nutrition-sensitive intervention, but lack of evidence on efficacy holds back its scalability $^{\text{[55]}}$. Our randomized controlled feeding trial provides the first evidence that consumption of yellow cassava can improve serum retinol concentrations of marginally vitamin A deficient children. Priority should be given to breeding and release of varieties that contain more β -carotene, in order to have a larger impact on vitamin A status.

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Abstract

Background: Biofortification of cassava with provitamin A can potentially reduce vitamin A deficiency in low-income countries. However, little is known about consumer acceptance of this deep yellow variety of cassava compared to the commonly available white varieties. We aimed to determine the sensory and cultural acceptability of the consumption of provitamin A rich cassava in order to identify key factors predicting the intention to consume provitamin A rich cassava by families with school-aged children in Eastern Kenya.

Methods: Sensory acceptability was measured by replicated discrimination tests and paired preference tests among 30 children (7–12 yr) and 30 caretakers (18–45 yr) in three primary schools. Cultural acceptability was assessed with a questionnaire based on the combined model of The Theory of Planned Behavior and The Health Belief Model in one primary school among 140 caretakers of children aged 6 to 12 years. Correlations and multivariate analyses were used to determine associations between summed scores for model constructs.

Results: Caretakers and children perceived a significant difference in taste between white and provitamin A rich cassava. Both preferred provitamin A rich cassava over white cassava because of its soft texture, sweet taste and attractive color. Knowledge about provitamin A rich cassava and its relation to health ('Knowledge' ((b = 0.29, p =.01)) was a strong predictor of 'Health behavior identity'. Worries related to bitter taste and color ('Perceived barriers 1' (b = 20.21, p = .02)), the belief of the caretaker about having control to prepare cassava ('Control beliefs' (b = 0.18, p = .02)) and activities like information sessions about provitamin A rich cassava and recommendations from health workers ('Cues to action'(b = 0.51, p \leq .01)) were the best predictors of intention to consume provitamin A rich cassava.

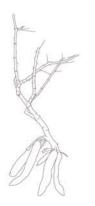
Conclusions: Provitamin A rich cassava is well accepted by school children in our study population.

Chapter 3

Biofortified cassava with provitamin A is sensory and culturally acceptable for consumption by primary school children in Kenya

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Introduction

Biofortification of crops can increase the micronutrient content using traditional breeding methods or modern biotechnology. Biofortification of staple foods with micronutrients is regarded as a sustainable approach to reduce micronutrient malnutrition. It could potentially benefit people in rural and remote areas with limited access to alternative possible interventions such as supplementation or introducing fortified food products^[1,2,3].

Vitamin A deficiency (VAD) is a public health problem among young children and pregnant women in low-income countries. Vitamin A is an essential micronutrient for maintaining eye sight, immune function, and growth and development. VAD is caused by low dietary intake of vitamin A in combination with malabsorption and high excretion of vitamin A due to common illnesses^[4]. In Kenya, the VAD prevalence is classified as severe by WHO with more than 60% of preschool children having moderate or severe VAD^[5].

Cassava (*Manihot esculenta* Crantz) is a starchy staple food that is consumed widely in tropical and subtropical Africa, Asia and Latin America. This drought resistant crop can grow on poor soils with little water and can be harvested when needed, providing households with an alternative when the harvest of other crops fails^[6]. Cassava cultivars that are naturally rich in provitamin A were identified by the International Centre for Tropical Agriculture in Colombia (CIAT). New cultivars with a total carotenoid content range of 100–10,000 µg/100 g in fresh cassava have been developed by selective breeding of cassava with high-carotene germplasm by CIAT and the International Institute of Tropical Agriculture (IITA) in Nigeria^[7]. These cultivars were introduced in Kenya for an efficacy trial conducted in 2012 among school aged children^[8].

Biofortification changes the color of cassava roots from white to deep yellow, due to the increase in provitamin A content. Not only appearance but also taste can be influenced due to lower dry matter concentration associated with higher provitamin A concentration^[7]. For biofortification programs to be successful, the biofortified crop needs to be accepted by both farmers and consumers^[9]. Consumer acceptance depends on the sensory characteristics and beliefs and practices in the community^[2]. Little is known about consumer acceptance of these new cultivars of cassava.

The Theory of Planned Behavior (TPB) and the Health Belief Model (HBM) are two psychosocial theories that are widely used in explaining food-related behaviors. According to the TPB, behavior is a conscious effort mediated by intention^[10] and in the HBM health behavior is expected to result from a set of core beliefs^[11]. A combination of these two theories has been shown to improve the ability to explain nutrition behavior^[12,13,14]. Most acceptability studies in developing countries are entirely qualitative and use for example focus group discussions and interview methods in study populations selected by convenience^[15,16], without verifying the associations between beliefs or attitudes and consumption in larger representative populations. As such, a lot more insight can be gained into possible ways to influence nutrition behavior. Willingness to pay studies^[17,18] are also used to assess acceptability of new foods, though not useful in our situation as our population depends mainly on their own production of crops.

In this study, we aimed to determine the sensory and cultural acceptability of the consumption of provitamin A rich cassava in order to identify key factors predicting the intention to consume provitamin A rich cassava by families with school-aged children.

Materials and methods

Ethic statement

Research authorization was given by the Ministry of Higher Education, Science and Technology, Kenya. The study was exempted from ethical approval by the Ethics and Research Committee of Kenyatta National Hospital in Kenya, because the study used non-invasive methods and because it was part of a larger study that had approval (P2293/07/2011). The study was explained to and written informed consent was obtained from the caretakers on behalf of their children before the study started.

Study area

The research was conducted in Kibwezi district of Eastern Province in Kenya, an arid to semi arid area (ASAL) where cassava is grown but not consumed daily. This research site was chosen based on eligibility for conducting an efficacy study with provitamin A rich cassava among primary school children at a later point in time.

Study participants and sampling

The study comprised two parts: 1) a sensory evaluation including a difference test and a preference test^[19,20], and 2) a cultural acceptability study using a questionnaire. Three public primary schools were chosen out of 15 eligible schools in the area, based on high prevalence of vitamin A deficiency measured in the previous year (unpublished results). For the sensory evaluation, 10 caretakers with a child between 6 and 12 years old were recruited in each of the three schools. For the cultural acceptability study, 140 caretakers participated in only one out of the three schools, due to exposure of the other two schools to information about provitamin A rich cassava the previous year. All caretakers of children in the age of 6 to 12 years were eligible and were only interviewed for one randomly chosen child if they had more than one eligible child. Any other eligible siblings were excluded, as dietary habits tend to be the same within one household. Data was collected over a period of four weeks in May 2011 by three trained enumerators for the sensory study and by six trained enumerators for the cultural acceptability study.



Study measurements

For the sensory study a provitamin A rich cassava variety (97/ 1170, cultivated by traditional breeding techniques) and a commonly available white cassava variety (Ex-Mariakani) were peeled and cooked for 30 minutes in water and manually mashed with a little oil and salt. Participants received instructions on the tests and a demonstration was given with mango and pineapple. The difference (or triangle) test consisted of six rounds per subject, each with three samples, of which one was different. Each round contained a randomly assigned, different serving order. Participants were blindfolded prior to serving the samples to prevent the color of the cassava from influencing the decision. The participant tasted the three samples and indicated to the enumerator which sample was the odd one. They were allowed to swallow the sample but had to rinse their mouths with water after each

taste. This test was based on the alternative hypothesis that the probability of the participant making the correct decision when perceiving a difference between samples had to be larger than one out of three (i.e. $Ha:Pt > 1/3)^{[19]}$).

The preference test was done after the difference tests with a randomly assigned serving order of the two cassava varieties. Participants were not blindfolded anymore, and were asked to taste the two samples, to choose the variety they preferred the most and to write down or mention the reason to the enumerator. They were allowed to swallow the sample but had to rinse their mouths with water after each taste. We hypothesized that participants would have a preference for one type of cassava (Ha: $P(A) \neq P(B)$)[20].

Cultural acceptability was assessed by a questionnaire based interview. The interview was conducted with caretakers of the children as children below 12 years of age may not provide reliable answers and are unable to handle scale scores^[21]. The questionnaire consisted of two parts; the first part aimed to collect information on general socio-demographic characteristics followed by a second part comprising statements related to the constructs of the combined TPB and HBM model (see Figure 1). For the latter, 67 statements were formulated based on findings in literature and a preparatory food ethnographic study on cassava and vitamin A deficiency, including a pile sort, a food attributes and differences study, and focus group discussions among caretakers and key informants[22, 23]. The statements were sorted into 12 constructs according to the model. Example statements per constructs are given in Table 1 and an explanation of the constructs is given in the footnote of Figure 1. Respondents were asked to indicate their level of agreement with a statement on a 5-point Likert scale, ranging from strongly disagree to strongly agree. Exceptions were made for the constructs 'Behavior' and 'Behavioral Intention' for which a time scale was used: never, once or less per month, 2-3 times per month, once a week, two or more times a week.

The constructs 'Knowledge' and 'Perceived Severity' were measured with a 5-point Likert scale with an additional column 'don't know'. Statements of all constructs were scored from 1 to 5 with the item 'don't know' being scored as neutral. However the constructs 'Behavior' and 'Behavioral intention' were scored 0 to 4. Questions within the constructs 'Attitudes towards behavior' and 'Subjective norms' consisted of a statement and an evaluation of that statement. Statements were scored 1 to 5 and evaluations were scored 22 to 2 and the two outcomes were multiplied, resulting in a score possibility of 210 to 10 for that statement. Questions for unmarried women within the construct 'Subjective norms' were recorded as not applicable and scored as neutral. The questionnaire was translated into Kiswahili and correctness was checked by back translating into English. The questionnaire was pretested, which resulted in small adjustments in the interpretation and explanation of the questions. After the interview the statements were scored and all scores within a construct were summed, resulting in a total construct score per respondent.

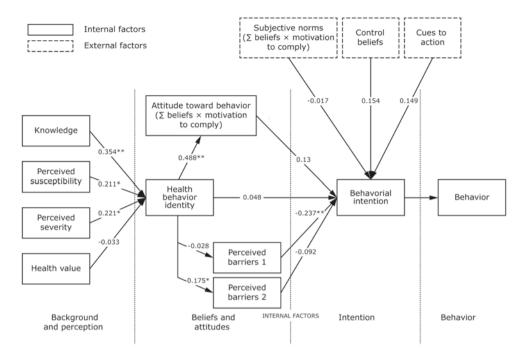


Figure 1. Correlations of the constructs using the combined health belief and theory of planned behavior models. (*Adjusted model based on Sun et al 2006*)^[14]

* p<0.05, ** p<0.001 (both two tailed) The model: The model is based on the idea that the construct Behavioral intention(intention to feed child with provitamin A rich cassava) is an important predictor for Behavior (feeding the child provitamin A rich cassava) The constructs related to 'Background and perception', are 'Knowledge' (about provitamin A rich cassava and VAD), Perceived susceptibility' (perception of developing VAD), 'Perceived severity' (notion of seriousness of developing VAD), and 'Health value' (notion of priority to stay healthy). The constructs related to 'Beliefs and attitudes', are 'Health behavior identity' (notion that it is good to eat vitamin A provitamin A rich cassava), 'Perceived barriers' (perceived obstacles which prevent the consumer from eating provitamin A rich cassava) and 'Attitude towards behavior' (positive or negative feeling towards eating provitamin A rich cassava). The external factors consist of the constructs 'Subjective norms' (perceived social pressure to consume provitamin A rich cassava), and 'Cues to action' (external triggers, which stimulate to consume provitamin A rich cassava).



Statistical Analyses

Data were analyzed with the Statistical Package for Social Science (IBM SPSS statistics 19.0). All statistical tests were 2-tailed and p values <0.05 were considered statistically significant. In the sensory evaluation, the responses for the difference test were not independent and probability of between and within person variance existed. This variance is known as over dispersion and is measured by gamma (γ), a value between 0 and 1. When γ is significantly greater than 0, the beta-binomial model has to be used to avoid an underestimation of the standard error. When γ is 0, there is no over dispersion and the binomial model can be used. To verify that γ was significantly greater than 0, Tarone's Z statistics^[24] was calculated to test the goodness of fit of the binomial distribution against the beta-binomial distribution. When Tarone's Z statistics is greater than 1.645 the

beta-binominal distribution has a better fit (a =0.05, one tailed). To identify the number of correct responses needed for a significant difference in taste the critical values by Lawless and Heymann^[19] were used for the binomial model, and those by Bi and Ennis^[25] for the beta-binomial model. For the preference test, only one answer was given per respondent and therefore the critical values by Lawless and Heymann^[20] were used to evaluate whether the minimal values for statistical significance were reached.

For the cultural acceptability evaluation, descriptive statistics were used to examine socioeconomic characteristics of the caretakers and to calculate median scores of the constructs. Multiple item constructs were tested for reliability and internal consistency using Cronbach a and item-total correlation[26]. Consistency within a construct is achieved when the Cronbach a is 0.7 or higher and when the corrected item-total correlation of all items in a construct is higher than 0.30. If exclusion of an item resulted in a higher Cronbach a value for the total item set, the item was excluded from the construct to enhance the consistency of the construct. The consistency within the construct 'Perceived barriers' was low (Cronbach a < 0.5). We separated the initial 16 statements into two distinct constructs: 'Perceived barriers 1' reflecting the worries about taste and appearance of provitamin A rich cassava (4 statements) and 'Perceived barriers 2' representing the believed facts about provitamin A rich cassava (12 statements). Within construct 'Perceived barriers 2' two statements were excluded to improve consistency. In total, 11 statements out of 67 were deleted from the initial questionnaire to improve reliability. Final Cronbach a values ranged from 0.56 to 0.80 which indicates medium to sufficient reliability of the constructs (Table 1). Median scores of the constructs were high in comparison to the range of scores which showed that caretakers tended to agree with the statements.

Spearman correlation was determined to assess bivariate associations between constructs. Multiple regression analyses was used to determine which constructs significantly predict the intention to consume provitamin A rich cassava. All models were adjusted for interviewer, age and education of the caretaker.

Table 1. Internal consistency and median scores of the constructs (n=140).

Construct	Number of items	Cronbach a	Median	Range of score*	Example statements
Knowledge	5	0.61	19	13 - 25	Vitamin A can prevent infections
Perceived susceptibility	2	0.69	10	2 - 10	School children are at risk of developing VAD
Perceived severity	6	0.8	26	10 - 30	Lack of vitamin A makes my child susceptible to diseases
Health value	2	0.56	10	4 - 10	That my child can properly see during dusk or dawn is the most important thing in my life
Health behavior identity	1	n/a	3	1 - 5	Eating provitamin A rich cassava is good for my child
Attitudes towards behavior	9	0.73	57	-13 - 80	a) Provitamin A rich cassava is nutritious b) I find it important to prepare food that is nutritious for my child
Perceived barriers - 1	4	0.96	13	4 - 20	I worry about the yellow color of provitamin A rich cassava
Perceived barriers - 2	10	0.60	40	16 - 50	Cassava is expensive to buy
Subjective norms	5	0.66	21	-20 - 50	a) My husband determines when to prepare cassava. b) The opinion of my husband on what to prepare is important to me
Control beliefs	3	0.72	15	9 - 15	I decide what food to buy for my household
Cues to action	8	0.80	36	11 - 40	School children in my household suffering from VAD would make me decide to buy provitamin A rich cassava
Behavioral intention	1	n/a	4	0 - 4	Will you prepare provitamin A rich cassava for your child in the future?

^{*}Range refers to minimum and maximum score for each construct in the study.

Results

In the sensory study a total of 30 caretakers (97% female), 31.6 ± 7.1 (mean \pm SD) years old, and 30 children (63% female), 8.9 ± 1.6 years old, participated independently. Respondent variability for the discrimination test was moderate for the adults group (y=0.17) and 0 for children (y=-0.01). Tarone's Z statistic was 3.6 for caretakers and -0.2 for children. Therefore the beta-binominal model was used for caretakers and the binomial model for children in the discrimination test. Both caretakers and children were able to detect a significant difference between provitamin A rich and white cassava: 180 difference tests were administered in both groups and 130 correct answers were observed for the caretakers and 89 for the children (Table 2). Both caretakers and children preferred the provitamin A rich cassava over the white cassava. Out of the 30 answers in both groups, 22 caretakers and 21 children had a preference for provitamin A rich cassava (Table 3). Characteristics attributed to provitamin A cassava were: attractive color, soft texture and sweet taste.

Of the 140 caretakers interviewed in the cultural acceptability study, the majority was female (96%), married (87%), between 18 and 50 years of age (83%) having received at least partly primary education (76%). White cassava was cultivated in their farms by 44% of the caretakers and 45% of them reported to have grown it in the previous season. Only 27% reported consumption of white cassava in the past month by their children. Only 15% of the caretakers had heard of provitamin A rich cassava before the interview but none were currently feeding it



to their children. Opinions of the caretakers concerning the different construct are described below per construct.

Table 2. Results for the difference test with provitamin A rich and white cassava.

Difference test	Caretakers (n=30)	Children (n=30)
Participant variability (y)	0.17*	0.00
Appropriate model	Beta-binomial	Binomial
Max. no. of correct responses	180	180
No. of correct responses needed for significance	75	71
No. of correct responses observed	130 **	89 **
Discrimination μ_0 Test: $\mu_0 = 1/3$	072	049

^{* =} significant (a = 0.05) using Tarone's Z statistic.

Construct 'Behavioral intention': Almost all caretakers had the intention to prepare provitamin A rich cassava for their children of which 64% were willing to do this two or more times per week. Construct 'Health behavior identity': Forty-seven percent of the respondents reported that eating provitamin A cassava would be good for their child, but 49% were neutral about this.

Construct 'Perceived barriers 1': Color, taste, texture and bitterness were worries for 44 to 49% of the caretakers.

Table 3. Results for the preference test with provitamin A rich and white cassava.

Preference test	Caretakers (n = 30)	Children (n = 30)
Appropriate model	Binomial	Binomial
Max. no. of responses	30	30
No. of responses needed for significance ($\alpha = 0.05$)	21	21
No. of responses observed favoring yellow cassava	22*	21*
Paired preference m Test: m0 = 1/2	0.73	0.70

^{* =} significant (a = 0.05).

Construct 'Perceived barriers 2': Most of the caretakers (96%) agreed that cassava peel is poisonous, the crop can easily be destroyed by wild animals (95%), the crop needs a lot of rain to grow (79%) or takes long to mature (71%), it is expensive (60%), not available in the market (56%) and that cuttings (planting material) are not available (61%). Furthermore most caretakers indicated that eating too much raw cassava can upset the stomach (95%) and can cause indigestion (74%).

Construct 'Cues to action': Most of the caretakers strongly agreed that information sessions about provitamin A rich cassava (98%), recommendations from health workers (98%) and provision of cassava cuttings (96%) would convince them to prepare provitamin A rich cassava for their children.

^{** =} significant ($\alpha = 0.05$).

Construct 'External control beliefs': Caretakers considered themselves to be in control to decide what food to prepare (98%), to buy (99%) for their households and to prepare cassava (99%) for their children. Construct 'Subjective norms': The child itself (76%) and health workers (64%) would mainly influenced the caretakers to prepare provitamin A cassava for their children. Opinions about food that were of importance to the caretakers were those of health workers (99%), the child itself (93%), the father (79%), community members (76%) and village elders (69%).

Construct 'Knowledge': Eighty percent of the caretakers did not know that provitamin A rich cassava contains vitamin A and is beneficial for the health of their child. Most agreed with the statement that cassava contains starch (82%). They also indicated that lack of vitamin A is associated with diseases (66%).

Construct 'Perceived susceptibility': According to the caretakers, school children in general (81%) and their own school children in particular (75%) were at high risk of developing vitamin A deficiency.

Construct 'Perceived severity': Most caretakers agreed that lack of vitamin A made a child susceptible to disease (89%), slowed the growth of the child (87%) and that vitamin A was associated with (night) blindness (69%).

Construct 'Health value': Most of the caretakers reported that it was important for them that their child has a good eye sight (99%) and can see during dusk or dawn (99%).

Construct 'Health behavior identity': Half of the caretakers (47%) agreed with the statement "Provitamin A rich cassava is good for my child", and the other half was neutral (49%).

Construct 'Attitudes towards behavior': Most of the caretakers indicated that cassava can be prepared in many different ways (98%) and that it is important to give food to their child that can be prepared in different ways (97%). Statements on the properties such as appearance and taste of the cassava were regarded by most of the caretakers as neutral (60–92%). Most of the caretakers indicated that it was important for their child to eat food that is nutritious (99%), sweet (98%), attractive (94%), satisfying (99%), and to eat food that makes the child strong and prevents diseases (99%). Correlations between the constructs are shown in Figure 1.



The constructs 'Knowledge', 'Perceived susceptibility' and 'Perceived severity' were significantly correlated with 'Health behavior identity', with the construct 'Knowledge' having the highest correlation (rs= 0.354, p<0.01). 'Health behavior identity' was significantly correlated with 'Attitudes towards behavior (rs = 0.488, p<0.01) and 'Perceived Barriers 2 (rs= 0.175, p< 0.05). 'Perceived Barriers 1' was the only construct significantly correlated with 'Behavioral Intention' (rs = -0.237, p<0.01) however in an inverse way. Only 'Cues to action' (rs =0.149, p= 0.07) and 'Control beliefs' (rs = 0.154, p= 0.078) were borderline significantly correlated with 'Behavioral intention'. The relative contribution of the constructs to the three models are shown in Tables 4, 5 and 6.

Table 4. Predictors of 'Health behavior identity' (model 1) to consume provitamin A rich cassava among the study population (n = 140).

Model and related constructs	Standardized Adj			Adjusted
	β	Р	R ²	R ²
Model 1 (Y = Health behavior intention)			0.30	0.23
Knowledge	0.29	< 0.01		
Perceived susceptibility	0.43	0.63		
Perceived severity	-0.01	0.96		
Health value	-0.14	0.07		

^{*}All models were adjusted for interviewer, age and education of the caretaker.

Table 5. Predictors of 'Behavioral intention' (model 2) to consume provitamin A rich cassava among the study population (n = 140).

Model and related constructs		Standardized		
	β	Р	R ²	R ²
Model 3 (Y = Behavioral intention)			0.17	0.09
Health behavior identity	0.18	0.07		
Attitude towards behavior	0.16	0.15		
Perceived barriers - 1	-0.21	0.02		
Perceived barriers - 2	-0.05	0.55		

^{*}All models were adjusted for interviewer, age and education of the caretaker.

Table 6. Predictors of 'Behavioral intention' (model 3) to consume provitamin A rich cassava among the study population (n = 140).

Model and related constructs		Standardized	d	Adjusted
	β	Р	R ²	R ²
Model 3 (Y = Behavioral intention)			0.25	0.18
Subjective norm	-0.09	0.32		
External control beliefs	0.18	0.02		
Cues to action	0.51	< 0.01		

^{*}All models were adjusted for interviewer, age and education of the caretaker.

Models were adjusted for interviewer, age and education of the caretaker which increased the variance of the models slightly (referred to as $adj\Delta R^2$. Our constructs explained only a small proportion of the variance in 'Health behavior identity' and 'Behavioral intention'. In the first model the constructs in 'Background and perception' explained 23% of the variance ($adj\Delta R^2$ =9%) in 'Health behavior identity', of which 'Knowledge' (β =0.29, p<0.01) was the only significant predictor and 'Health Value' was borderline significant (β =-0.14, p=0.07) (Table 4). The next two models looked at internal (model 2, Table 5) external (model 3, Table 6) predictors of 'Behavioral intention'. Nine percent of the variance ($adj\Delta R^2$ =1,9%) was explained by internal factors with 'Perceived barriers 1' as the only significant predictor (β =-0.21, p<0.05) and with 'Health behavior identity' as borderline significant (β =0.18, p=0.07). Eighteen percent of the variance ($adj\Delta R^2$ =0.05%) was explained by external factors with 'External control beliefs' (β =0.18, p=0.02) and 'Cues to action' (β =0.51, p<0.01) as significant predictors.

Discussion

We found that both caretakers and children were able to blindly taste a difference between the two types of cassava. When unblinded, they preferred the provitamin A rich cassava over the white cassava, indicating that provitamin A cassava is sensory acceptable. Moreover, almost all caretakers had the intention to prepare provitamin A rich cassava for their children; this intention was most strongly associated with 'Perceived barriers 1', 'Knowledge', 'External control beliefs' and 'Cues to Action'.

This study has some limitations. Firstly, caretakers for the sensory study were selected by the headmaster of the primary school based on willingness, role in the school and availability. This purposive selection may have resulted in inclusion of caretakers with a certain liking for cassava, or who were instructed as such and this might have contributed to a preference of provitamin A rich cassava. Secondly, the combined model of HBM and TBP has been previously used by others to assess factors that contribute to the consumption of a certain food^[12,13,14].

It is assumed that intention leads to behavior, however in our study this assumption could not be confirmed as the behavior of eating provitamin A rich cassava does not exist in our study population. To our knowledge, this is the first time the model is used for a biofortified food not yet available in the community. Thirdly, in the cultural acceptability study for the constructs 'Knowledge' and 'Perceived severity' the answer category "don't know" was coded as neutral. We assumed that caretakers would have answered 'neutral' if they would have had knowledge on the issue asked for. However, this assumption could not be verified and might have influenced the associations of especially 'Knowledge' (46% of caregivers replying 'don't know') and 'Perceived severity' (19.7% replying 'don't know') with health behavior identity. Furthermore the mean construct scores were generally high and correlations between constructs and the variance explained by the models was low. Caretakers may have found it difficult to disagree with the statements. A strong intention to perform a certain behavior can lead to lower correlations between constructs and low variance in the sample population as compared to the general population^[27]. We anticipated this and used a 5-point Likert scale instead of a 3-point Likert scale in order to allow for more options within the agreement statements. Low correlation and low variance does not necessarily weaken the effect and importance of the significant predictors to the dependent variable.



Similar studies also showed a low correlation and low variance^[12,13,14]. This may imply that there are other factors contributing to the unexplained variance by the models that are left unexplained. Including a measure of anticipated effect (beliefs about whether or not feelings of regret or upset will follow from not performing the behavior) may help in reducing part of the unexplained variance^[28].

Children in our study were less able to discriminate between the provitamin A rich cassava and the white cassava than caretakers (89 versus 130 correct answers). This may be due to lower memory skills of young children affecting their ability to remember a succession of flavors presented for evaluation in a sensory test^[21]. Training of the children on how to conduct the tests were held at the schools to improve their performance. Due to time limitations only 30% of the children were trained, which might have contributed to their lower ability to discriminate.

A general concern with introducing vitamin A biofortified staple foods is the change in sensory characteristics of the crop influencing its acceptability by the target

population. This does not seem to be a problem for orange fleshed sweet potato as shown in an effectiveness study in Mozambique in which the crop was widely consumed by the community^[29]. A study on the acceptability of provitamin A rich yellow maize in Zimbabwe revealed that the yellow maize is perceived as being inferior to white maize. The reason given for the inferiority was that most people received yellow maize as food aid giving yellow maize a negative perception. Authors also stated that due to the higher concentration of oil, fructose and carotenoids in yellow maize chemical changes can easily produce unacceptable organoleptic properties (bad taste) when poorly handled during transportation^[30]. Provitamin A rich cassava has no history with food aid. We found that the color and taste of provitamin A rich cassava are perceived as attractive and sweet and yellow cassava was therefore liked more than white cassava, especially by children. There is also some evidence that the carotenoids protect the cassava against post-harvest deterioration and therefore provitamin A cassava can be stored longer than white cassava^[31].

Our study population does not consume cassava on a regular basis, with only 27% of the children having consumed cassava in the previous month and 45% of the caretakers currently cultivating cassava on their farms. Since previous experience of consumers influences perception and beliefs, a population that consumes white cassava on a daily basis will probably have stronger beliefs towards the attributes of provitamin A rich cassava. Populations with a higher and more frequent intake of white cassava might be more reluctant towards provitamin A rich cassava than populations with a low and infrequent intake^[32]. In our study, the yellow color of the biofortified cassava was perceived as being attractive, but it may be possible that people in areas where white cassava is frequently consumed do not share this opinion.

'Knowledge', 'Perceived susceptibility' and 'Perceived severity' were all significantly correlated with 'Health behavior identity' in our study. However, when combined in one regression model, only 'Knowledge' remained a significant predictor of 'Health behavior identity'. Caretakers know about the risks of VAD and the severity of VAD, and also agree that their children are at high risk for VAD. However they are not aware of the link between consumption of provitamin A rich cassava and reducing VAD. Studies on fonio consumption in Mali^[12] and amaranth grain in Kenya[13] also found that 'Knowledge' was a strong predictor of 'Health behavior identity' and this may be because the target population is not yet aware of the extra nutrition and health benefits of the targeted food. Therefore it is important to create awareness about the relation of consuming provitamin A rich cassava and reducing VAD. Comparable research projects concentrating on yellow maize and orange-fleshed sweet potato, confirm that educational sessions and awareness campaigns are effective in increasing consumer acceptability of biofortified crops[30,33,34]. A model often used in behavior change research is the transtheoretical model, also called the Stages of Change Model [35]. This model suggests that health behavior change consists of five distinct stages; pre-contemplation, contemplation, preparation, action, and maintenance. Our population can be considered as being in the pre-contemplation or the contemplation stages; although they are aware of the vitamin A deficiency problem, they do not know that consuming provitamin A rich cassava can contribute to a reduction of VAD. Therefore, they may not feel the need or have commitment to incorporate vitamin A rich cassava in their diet. Providing them with knowledge about the relation between vitamin A intake and provitamin A rich cassava might bring them to the next stage of preparation to consume provitamin A rich cassava.

The internal factor 'Perceived barriers 1' (related to worries about bitter taste and color) significantly predicted 'Behavioral intention'. Provitamin A rich cassava is a new cassava variety and it appears that caretakers worry about the taste and appearance of the new cassava. We found in the sensory study that provitamin A rich cassava had a sweet taste (as opposed to bitter) and the color was attractive. We could speculate that these barriers can be taken away if people can taste and see the provitamin A rich cassava.

'Control beliefs' and 'Cues to action' were significant predictors of intention in the multivariate analysis. 'Cues to action' was also found to be a predictor of consumption of amaranth grains in Kenya[13] and of iron-fortified soy sauce in China^[14]. However, in these studies, 'Control beliefs' was not a predictor. In our community the caretakers feel that they have control over the decision to prepare provitamin A rich cassava for their children. It is unlikely that a caretaker performs a behavior that is outside their control[36] and therefore our finding suggests that when the caretaker is in control of buying and cooking provitamin A rich cassava, she will have the intention to prepare it for their children. We found that 'Subjective norm', reflecting social pressure, is not a predictor of intention to consume provitamin A rich cassava in this population. It might be that social pressure is not an issue in our community regarding food choice, particularly cassava. This was also seen in other studies and it was even suggested to be taken out of the model[14,37]. It could be that staple foods that are generally consumed are well accepted in the community and therefore normative beliefs regarding consumption are low. It has been shown by some studies focusing on sensitive behavior such as binge drinking^[38], smoking^[39] and driving violations^[40], that subjective norms strongly predicted behavior.

Our goal was to determine predictors of consumption of provitamin A rich cassava in order to be able to plan the introduction of this biofortified cassava in a good and successful way. Overall, we found that the yellow color of provitamin A rich cassava is no barrier for consumption in our research population. We found that almost all caretakers had the intention to prepare provitamin A rich cassava for their children and that this intention can be increased by:



- 1. reducing barriers like worries about color, taste, texture and bitterness;
- 2. increasing knowledge on vitamin A deficiency and provitamin A rich cassava;
- 3. empowering mothers to make the decisions for the household on what to cook;
- 4. involving health workers in the promotion of consumption of provitamin A rich cassava through information sessions about provitamin A rich cassava for caretakers.

These initial findings are encouraging, since they underline the potential of this biofortification strategy to become successful. Similar studies in other populations should reveal whether our findings can be extrapolated to a wider scale.

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Abstract

Background: The reference method to assess vitamin A status is serum retinol concentration determined by high-performance liquid chromatography (HPLC). This method is expensive, technically demanding and rarely available in developing countries. Our objective was a) to assess the diagnostic performance of proxy markers in detecting vitamin A deficiency; and b) to derive decision rules based on these markers to estimate vitamin A deficiency prevalence.

Methods: A survey was conducted in 15 rural primary schools in Eastern Province, Kenya with 375 children aged 6–12 years (25 randomly selected per school). Serum retinol concentration <0.70 μ mol/L by HPLC was used to define vitamin A deficiency. Proxy markers for vitamin A deficiency were serum concentrations of retinol binding protein (RBP), transthyretin, retinol measured by fluorometry and RBP:transthyretin molar ratio.

Results: Vitamin A deficiency prevalence (HPLC) was 18%. Transthyretin and RBP showed the best diagnostic performance individually, with area-under-the-curve (AUC) values of 0.96 and 0.93. When combined, and with C-reactive protein added, the AUC increased to 0.98. A simple decision rule $\{(-15.277 \times [RBP, \mu mol/L] - 7.013 \times [Transthyretin, \mu mol/L] + 0.367 \times [C-reactive protein, mg/L] + 24.714) > -0.237\}$ yielded an unbiased estimate of the prevalence of vitamin A deficiency.

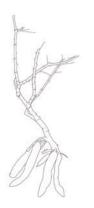
Conclusions: The combination of transthyretin, RBP and C-reactive protein could eventually replace retinol by HPLC in resource-poor settings as the preferred method to assess the population burden of vitamin A deficiency.

Chapter 4

Proxy markers of serum retinol concentration, used alone and in combination, to assess population vitamin A status in Kenyan children

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submitted

Introduction

Serum retinol (vitamin A) concentration is considered to be the reference method for assessment of vitamin A status, and is recommended by the World Health Organization to assess vitamin A deficiency in national health surveys^[1]. Its measurement requires high-performance liquid chromatography (HPLC), which is expensive, technically demanding and rarely available in developing countries^[2]. In addition, measurement of serum retinol requires large serum volumes that can only be obtained by venipuncture, and must be stored in tubes impermeable to light until laboratory analysis.

Several serum indicators proposed as proxy markers of vitamin A status may be conveniently used in resource-poor settings. These include retinol binding protein (RBP) concentration, the molar ratio of RBP: transthyretin, and retinol concentration measured by fluorometry[3]. RBP is a transporter protein that binds, transports and delivers retinol to target organs. Its secretion from the liver, where it is produced, into circulation depends on circulating retinol levels^[4]. Studies in rats suggest that RBP is present in serum in a 1:1 molar ratio to retinol, but surveys in humans indicate that this ratio can be higher and is influenced by inflammation, protein-energy malnutrition, obesity, vitamin A status, iron status and pregnancy^[5]. Transthyretin is involved in transport of retinol through the formation of a complex with RBP and retinol, which prevents the glomerular filtration of the RBP molecule in the kidneys^[6,7]. The molar ratio of RBP: transthyretin has been proposed as an indicator of vitamin A status unaffected by inflammation[8]. Both RBP and transthyretin can be measured relatively easily by enzyme-linked immunosorbent assay (ELISA). Fluorometry exploits the characteristic of retinol to fluoresce under influence of ultraviolet light, particularly when bound to RBP[9], allowing its measurement using a point-of-care test under field conditions.

To our knowledge, no studies have evaluated the diagnostic performance of combinations of these proxy markers to assess vitamin A status, and few studies considered the effect of diagnostic error on prevalence estimates of vitamin A deficiency. The present study, conducted among Kenyan children, aimed to assess the diagnostic performance of the proxy markers listed above, alone or in combination, in detecting vitamin A deficiency defined as serum retinol concentration <0.70 µmol/L (measured by HPLC). In this analysis, we considered inflammation markers, age, body mass index for age z-score and iron status as additional diagnostic markers. Secondly, we aimed to derive decision rules based on these markers to estimate the prevalence of vitamin A deficiency.

Materials and methods

Subjects and sample collection

The study was approved by ethical committees in Kenya and the Netherlands. We conducted a survey (June 2010) at 15 primary schools in Kibwezi and Makindu districts in Eastern province, Kenya, which had been selected from 45 public schools based on size (>350 children aged 6–12 years) and having no school feeding program. For each school, we randomly selected 25 children from an enrolment list of all children aged 6–12 years (n=375), and included those who were apparently healthy and without fever (ear drum temperature <37.5°C) upon examination by the research physician, and whose guardians had provided prior informed consent. Venous blood (6 mL) was obtained from each fasted child and

kept shielded from light at 2–8°C for 30–60 minutes. After centrifugation (1200 g, 10 minutes), serum was kept for 4–8h at 2–8°C and subsequently stored in liquid nitrogen (–196°C) in Kenya, and at –80 °C during transport and storage in the Netherlands. Blood samples were obtained by finger prick to measure hemoglobin concentration (HemoCue, Ängelholm, Sweden). Weight and height were measured according to WHO guidelines^[10] to the nearest 0.1 kg and 0.1 cm using a mechanical floor scale and a portable stadiometer (Seca, Hamburg, Germany).

Biochemical analyses

Concentrations of retinol (by HPLC), RBP and ferritin were determined at Wageningen University, the Netherlands (August 2010). Samples used to measure retinol concentrations were processed under subdued yellow light.

We added 200 µL sodium chloride (0.9% w/v in water) and 400 µL 96% ethanol, containing retinyl acetate as an internal standard, to 200 µL serum. Serum samples were extracted twice with 800 µL hexane for 5 minutes using a horizontal laboratory shaking machine at 250 reciprocations per minute (Edmund Buehler SM25, GmbH, Heckingen, Germany) and next centrifuged for 2 minutes at 3000 g. The hexane supernatants were pooled into an HPLC vial. Twenty-five µL of the extract was injected directly into a polar BDS Hypersil CN HPLC column (150×3 mm inner diameter, particle size 5 µm) with a Javelin NH2 quard column (both from Keystone Scientific, Bellafonte PA, USA). The HPLC system (Spectra, Thermo Separation Products Inc., San Jose CA, USA) was equipped with two pumps (model P2000), a solvent degasser (model SCM400), a temperature-controlled auto sampler (model AS3000), an UV-visible forward optical scanning detector (UV3000), interface (model SN4000), and control and integration software (Chromquest 5.0). As eluent, we used a mixture of hexane-isopropanol (98.5%:1.5% v/v) containing triethylamine (0.1% v/v) as a mobile phase additive to reduce peak tailing, at a constant flow of 0.7 mL/min, Separations were measured at 325 nm and quantified using the internal standard method against retinol standards. Total runtime was 5 min. Within-run and between-run CVs were 1.6% and 2.1%, respectively, based on in-house control serum. Analysis of standard reference material SRM 968e from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) revealed deviations of 0.3%, 0.2% and 5% from certified values for the low, medium and high levels (1.19 µmol/L, 1.68 µmol/L and 2.26 µmol/L, respectively). Duplicate measurements were done on 10% of samples, resulting in a mean CV of 2.0%.

RBP concentrations were determined by immunoassay (catalogue DRB400, Quantikine, R&D Systems, Minneapolis, USA). Results were read in duplicate for 10% of samples. The inter-plate CV for six plates was 10.4%. The intra-assay CV for duplicate samples was 6.0%.

Ferritin concentrations were determined by enzymatic immunoassay (Ramco Laboratories, Stafford, Texas). Results were read in duplicate for 10% of samples. The inter-plate CV for 6 plates was 8.8%. The intra-assay CV for duplicate samples was 9.7%.

A point-of-care fluorometer (iCheck™ FLUORO; BioAnalyt, Teltow, Germany) was validated (Supplement 1) and used (September 2011) to measure concentrations of vitamin A (retinol and retinyl palmitate) at excitation and emission wavelengths of 330 nm and 470 nm. Children were ranked on serum retinol concentration and a subset of 105 samples was selected by taking every third sample. If the



sample was insufficient, the next sample on the list was taken to ensure the same concentration range. 250 μL of serum was injected into a sealed glass cuvette prefilled with a proprietary reagent (iEx $^{\text{TM}}$ MILA, BioAnalyt) comprising a mixture of alcohols and organic solvents. 250 μL phosphate buffered saline solution (PBS) was added to obtain the required 500 μL sample volume and the result multiplied by two. Samples were measured according to manufacturer guidelines. Control samples provided by manufacturer were measured at the beginning and end of each batch of measurements, and were within the expected range.

Serum concentrations of transthyretin, C-reactive protein and a1-acid glycoprotein were determined by immunoturbidimetric assays on a Cobas Integra 800 system (Roche Diagnostics, Mannheim, Germany) at University Medical Centre, Leiden, the Netherlands (October 2010). Transthyretin concentration was measured using the PREA assay (Roche), with CVs of 1.9% and 3.2% at concentrations of 4.7 μ mol/L and 11.4 μ mol/L. C-reactive protein concentration was measured by Tinaquant ultrasensitive assay (Roche), with CVs of 1.8% and 1.9% at concentrations of 3.98 mg/L and 12.81 mg/L. a1-Acid glycoprotein concentration was measured using the Tina-quant AAGP2 assay (Roche), with CVs of 1.3% and 0.5% at concentrations of 0.77 g/L and 1.27 g/L.

Statistical analyses

Anthropometric z-scores were calculated using Anthro-plus (WHO, version 3.2.2). Results were analyzed using statistical software packages IBM SPSS 20.0 and STATA 12. Comparisons were done separately for all children and for those without inflammation, defined as serum concentrations of C-reactive protein <5 mg/L or a1-acid glycoprotein <1 g/L^[11]. Distributions of serum markers were inspected by visual examination of histograms, and were described using conventional methods. We defined vitamin A status by serum retinol concentration (HPLC) < 0.70 µmol/L (deficient) or ≥ 0.70 µmol/L (replete). Scatter plots and linear regression analysis were used to assess linearity in associations of the proxy markers with serum retinol concentration. Receiver Operating Characteristics (ROC) curves were used to assess the diagnostic accuracy of proxy serum markers in detecting vitamin A deficiency, whether alone or in linear combinations in comparison with retinol by HPLC. Diagnostic accuracy by visual inspection of these curves and by assessing differences in the AUC with corresponding p-values was compared. A Bland-Altman plot was used to assess the agreement between measuring retinol concentration by HPLC and fluorescence^[12].

Combinations of proxy markers may have better ability than single markers to distinguish between children with and without vitamin A deficiency. For pairs of markers, we assessed this distinguishing ability by visual inspection of scatter plots, with individuals being classified by vitamin A status. Logistic regression was used to assess the added diagnostic value of each marker and to produce linear predictors (combinations of diagnostic test results), which can be interpreted as decision rules to classify vitamin A status. Each newly defined linear predictor was used to compute the probability of vitamin A deficiency for all subjects, which can be considered on its own as the quantitative outcome of a new, stand-alone diagnostic test. Thus we produced ROC curves by allowing this probability to vary within the range [0,1]). Using a stepped-forward selection procedure, we started the model with the best proxy marker when used alone, and successively added other proxy markers, serum markers of inflammation, age, body mass index-for-age z-score and iron status as explanatory variables. We settled on a

parsimonious model that only included markers found to have independent diagnostic value when used in combination with others, as judged by p-values for logistic regression coefficients. First, we assessed the goodness of fit of the model by plotting the predicted against the true observed vitamin A deficiency cases, with values grouped in deciles based on increasing predicted values. We assessed the ability of the model to discriminate between children with or without vitamin A deficiency by means of an ROC plot and its AUC. With this model, we calibrated the value of the linear predictor to produce an unbiased estimate of the prevalence of vitamin A deficiency. Given a diagnostic test with a binary outcome, a set of paired values for sensitivity and specificity exists, that leads to a prevalence estimate that is identical to the true prevalence (Supplement 2). The intersection of this set and the ROC curve obtained with our parsimonious logistic regression model indicates the value of the linear predictor (and thus the diagnostic decision rule) that would lead to unbiased estimation of the prevalence of vitamin A deficiency. We calibrated the linear predictor to give unbiased estimates of the prevalence of vitamin A deficiency, with true prevalence arbitrarily selected as 6% and 15%, the mid-points for the ranges that indicate mild and moderate public health problems (2%-10% and 10%-20%, respectively)[13]. Similarly, we used 30% and 40% as an arbitrarily selected prevalence in the range (>20%) indicating a severe public health problem.

Results

Complete data were collected for 372 children; for three children, no blood sample could be obtained. Table 1 shows the characteristics of the total study population and the subsample (n=105) for which retinol concentration was measured by fluorometry. Vitamin A deficiency occurred in 18% of children, whilst 36 (9.6%) children had inflammation as indicated by elevated concentrations of C-reactive protein, a1-acid glycoprotein or both. Serum retinol concentration (HPLC) in the subsample was similar to the total sample (0.87 μ mol/L), and retinol measured by fluorometry was 0.79 μ mol/L. Excluding children with inflammation resulted in slightly increased concentrations of retinol (HPLC), RBP, transthyretin and retinol (fluorometry), a similar RBP: transthyretin molar ratio and a slight reduction in prevalence of vitamin A deficiency (15%).



Table 1. Characteristics of the study population

Main study	All children	Children without inflammation ²
n	372	336
Age, years	9.2 (1.9)	9.2 (1.9)
Sex, girls: boys	199:173 (54%:46%)	180:146 (54%:46%)
Serum retinol concentration by HPLC, µmol/L	0.87 (0.19)	0.88 (0.18)
Vitamin A deficiency ¹	68 [18%]	49 [15%]
Serum RBP concentration, µmol/L	0.67 (0.17)	0.68 (0.17)
Serum transthyretin concentration, µmol/L	3.0 (0.62)	3.0 (0.60)
RBP: transthyretin molar ratio	0.23 (0.04)	0.23 (0.04)
Serum C-reactive protein concentration >5 mg/L	17 [5%]	0 [0%]
Serum a 1-acid glycoprotein concentration > 1 g/L	33 [9%]	0 [0%]
Inflammation ²	36 [10%]	0 [0%]
Hemoglobin concentration, g/L	130 (11)	129 (12)
Serum ferritin concentration, µg/L (median, IQR)	19.8 (12.8, 30.2)	19.2 (12.0, 29.5)
BMI for age z-score	-1.29 (0.91)	-1.29(0.89)
Sub-study		
n	105	94
Age, years	8.9 (1.9)	8.9 (1.9)
Sex, girls: boys,	64:41 (61:39)	58:36 (62:38)
Serum retinol concentration by HPLC, µmol/L	0.87 (0.18)	0.88 (0.17)
Serum retinol concentration by fluorescence, $\mu\text{mol}/L$	0.79 (0.30)	0.79 (0.30)
Serum C-reactive protein concentration >5 mg/L	4 [4%]	0 [0%]
Serum a1-acid glycoprotein concentration >1 g/L	10 [10%]	0 [0%]
Inflammation ²	11 [11%]	0 [0%]
Hemoglobin concentration, g/L	130 (10)	130 (10)
Serum ferritin concentration, µg/L (median, IQR)	18.9 (11.4, 29.4)	18.2 (11.2, 27.7)
BMI for age mean z-score	-1.43 (0.91)	-1.43 (0.89)
(00) [01]		

Values indicate mean (SD) or n[%] unless indicated otherwise.

In univariate analysis, retinol measured by HPLC was strongly associated with RBP and transthyretin, and to a lesser degree with retinol measured by fluorometry and the RBP: transthyretin molar ratio (Figure 1).

Figure 2 shows the ROC plots for each proxy marker. The area under the curve was the highest for transthyretin and RBP (0.96 and 0.93, respectively), followed by retinol by fluorometry (0.81) and RBP: transthyretin molar ratio (0.56). Excluding children with inflammation resulted in a slight decrease in AUC for RBP and a slightly higher AUC for transthyretin and retinol by fluorometry, but did not appreciably change for the RBP: transthyretin molar ratio.

The Bland-Altman plot shows a mean difference of 0.083 μ mol/L between the HPLC and fluorescence methods, with limits of agreement of -0.40 μ mol/L and 0.57 μ mol/L. Results of the two methods diverted with serum retinol concentration, indicating that the fluorescence method tended to overestimate concentrations (Figure 3).

Logistic regression resulted in a model of vitamin A deficiency dependent on RBP, transthyretin and C-reactive protein and a linear predictor of

¹ Serum retinol concentration (HPLC) <0.70 µmol/L

 $^{^2}$ Defined by serum concentrations of C-reactive protein > 5 mg/L or a1-acid glycoprotein > 1 μ g/L

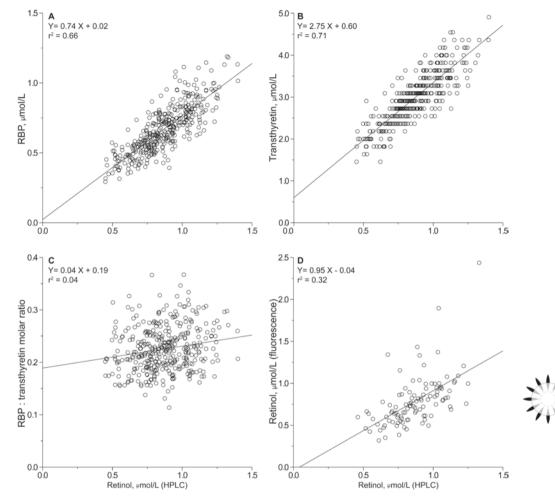


Figure 1: Associations of four proxy markers with serum retinol concentration (HPLC)

A: RBP, B: transthyretin, C: RBP: transthyretin molar ratio, D: retinol by fluorescence

(-15.277×[RBP μ mol/L] - 7.013×[Transthyretin μ mol/L] + 0.367×[C-reactive protein mg/L] + 24.714). Figure 4 illustrates that the observed versus the predicted probability of vitamin A deficiency were close to the line of identity, showing an excellent fit of the model. When used in combination, RBP and transthyretin were better at discriminating between children with and without vitamin A deficiency than when transthyretin was used alone (AUC: 0.98 versus 0.96; p=0.01) or when RBP was used alone (AUC: 0.98 versus 0.93; p=0.001) (Figure 2). Addition of C-reactive protein into the RBP and transthyretin model resulted in a marginal improvement of AUC but did not improve the model (AUC: 0.982 versus 0.979; p=0.44). Figure 5 shows the decision rules for unbiased estimates of the prevalence of vitamin A deficiency, at true prevalence values of 6%, 15%, 30% and 40%, and the corresponding sensitivity and specificity values.

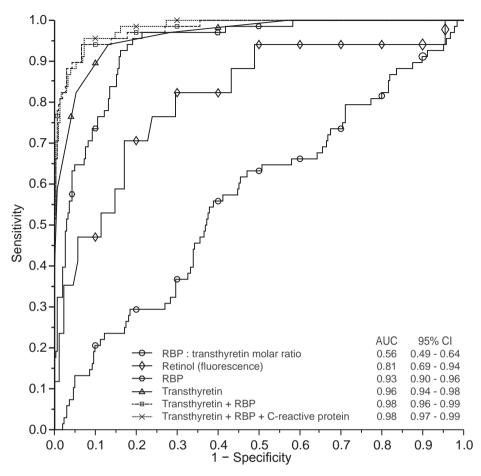


Figure 2: ROC curves for proxy markers of vitamin A deficiency

Discussion

Serum concentrations of transthyretin and RBP, when used alone, performed well in discriminating between children with and without vitamin A deficiency. Test performance was even better when these markers were used in combination, and addition of serum C-reactive protein concentration could lead to further improvement albeit marginally. We have shown how these three markers can be combined to estimate the prevalence of vitamin A deficiency in population surveys, based on a simple decision rule to determine individual vitamin A status.

Strong points of our study are: a) the novel use of combinations of multiple markers to determine vitamin A status; b) the relatively large sample size; c) the study population concerned children for whom vitamin A status is to be determined (as opposed to children with signs or symptoms suggesting deficiency, which may lead to biased estimates of the diagnostic performance); d) it went beyond an assessment of diagnostic accuracy as indicated by sensitivity and specificity but

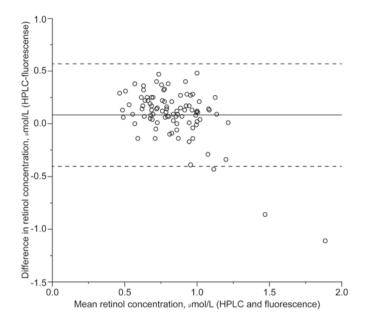


Figure 3: Bland-Altman plot of retinol by HPLC versus fluorescence

demonstrated the application of a diagnostic strategy using these markers for public health purposes (estimation of the prevalence of deficiency).

Although transthyretin has been used as a vitamin A marker in many studies, it has mostly been analyzed as the RBP: transthyretin molar ratio. Only one study reported the diagnostic performance of transthyretin[13], but was based on univariate analysis and used the relative dose-response test as a reference, which earlier has been questioned as valid indicator of vitamin A status[14]. Our results suggest that 2-3 proxy markers (serum concentrations of RBP, transthyretin and C-reactive protein) could replace serum retinol concentration measured by HPLC, with the advantages that these markers can be conveniently measured at relatively low cost by separate or multiplex ELISAs and require only a small blood volume collected by finger puncture. Although transthyretin seems stable at refrigerated or frozen conditions for up to several weeks[15], additional studies are required to assess its stability under field conditions. Although serum C-reactive protein concentration had limited diagnostic utility in this study, we note that it may be important in populations with more severe degrees of inflammation. Our results indicate that the RBP: transthyretin molar ratio is inferior and should not be used. Serum retinol concentration measured by fluorometry is also inferior but its diagnostic utility may need re-assessment if the technology can be improved.

Selection of cut-points for dichotomized diagnostic tests should depend on diagnostic aims. Vitamin A deficiency is defined by serum retinol concentrations $<\!0.70~\mu\text{mol/L}$ because individuals who meet this criterion are considered to be at increased risk of morbidity and mortality $^{[16]}$. To avoid missing cases, it may be desirable for a diagnostic test to have a high sensitivity in detecting such individuals, even at the expense of specificity. Another approach can be to maximize accuracy, i.e. the probability that individuals with and without vitamin A deficiency are correctly classified, which is appropriate if a false negative is considered to be equally undesirable as a false positive. In the present paper, our diagnostic aim was to



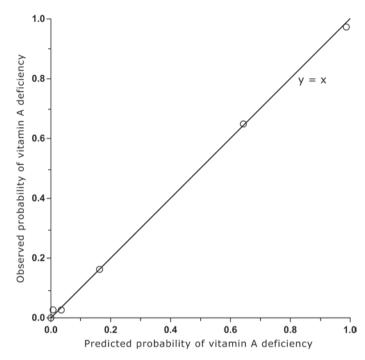
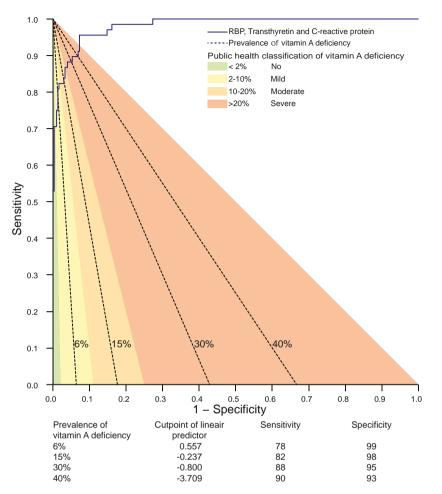


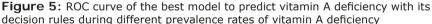
Figure 4: Goodness of fit of the model in predicting vitamin A deficiency

Predicted probability values are grouped in ten equal deciles based on increasing predicted probability values. Out of these 10 groups, only six can be seen as four groups contain the same predicted probability of 0 and overlap each other in this figure.

produce unbiased estimates of the prevalence of vitamin A deficiency. Selection of cut-points to maximize either sensitivity or accuracy will lead to overestimates of the true prevalence. When the true prevalence of vitamin A deficiency is low, the validity of the estimate depends almost entirely on specificity, and the optimal cut-point is one for which specificity is increased even at the expense of sensitivity.

These principles are illustrated in figure 5, which shows theoretical conditions whereby combinations of values for sensitivity, specificity and true prevalence give unbiased prevalence estimates (straight lines). However, the paired values of sensitivity and specificity that can be actually achieved with the combined use of three proxy markers (RBP, transthyretin and C-reactive protein) is indicated by the ROC curve. The intersect of the ROC curve and the straight lines determine the cut-point for the linear predictor that gives an unbiased prevalence estimate. Interpretation of this linear predictor is relatively straightforward. For example, at a true prevalence of vitamin A deficiency of 15%, individuals for whom (-15.277×[RBPµmol/L] - 7.013×[Transthyretinµmol/L] + 0.367×[C-reactive proteinmg/L] + 24.714) > -0.237 should be classified as deficient, whereas all others can be classified as not-deficient (in this formula, concentrations are indicated in straight brackets, and expressed in units as indicated). Such classification may serve as the basis to compute the prevalence estimate.





True prevalence was arbitrarily selected as 6%, 15%, 30% and 40% as the mid-points for the ranges that indicate mild, moderate and severe public health problems (2%-10% and 10%-20%, >20% respectively). Note that, with the true prevalence decreasing, the optimal cut-point for the linear predictor to provide an unbiased prevalence estimate results in an increased specificity even at the expense of sensitivity. Example, at a true prevalence of vitamin A deficiency of 15%, individuals for whom (-15.277×[RBP μ mol/L] - 7.013×[Transthyretin μ mol/L] + 0.367×[C-reactive protein mg/L] + 24.714) > -0.237 should be classified as deficient, whereas all others can be classified as not-deficient (in this formula, concentrations are indicated in straight brackets, and expressed in units as indicated



We arbitrarily selected prevalence values of 6%, 15%, 30% and 40% as the mid-points for the ranges that indicate vitamin A deficiency as a mild, moderate or severe public health problem, and allowed the optimal cut-point for our linear predictor to vary accordingly. These cut-points enable national surveys to assess population vitamin A status at a lower cost and with more accuracy. Further research is needed to confirm whether this linear predictor yields similar results in different populations and laboratories.

We conclude that the combination of transthyretin, RBP and C-reactive protein showed excellent diagnostic performance in assessing vitamin A status, and has great potential to eventually replace serum retinol concentration measured by HPLC as the preferred method to assess the population burden of vitamin A deficiency. Our methodology can be widely applied for other diagnostic aims.

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Supplement 1

Validation of fluorescence method to measure serum retinol concentrations

A first prototype of the fluorescence machine (iCheck) was tested in the field during sample collection in 2010. The correlation between serum retinol by HPLC and fluorescence was low (r=0.43 p<0.01), and sensitivity and specificity of fluorescence in detecting vitamin A deficiency (defined as serum retinol<0.70 μ g/L as analyzed by HPLC) were 69% and 55% (data not shown). Based on our findings and recommendations, the manufacturer made adjustments to the machine and provided an improved iCheck version in 2011. This supplement shows the methods and results of a validation of the 2011 iCheck in our laboratory under controlled conditions.

For this, 15 ml whole blood was obtained from 3 individuals and 3 aliquots of 5 ml were kept in the dark at 2° - 8° C. Two aliquots were centrifuged at 3000 g for 10 minutes and serum was stored at 2° - 8° C until analyses. For measuring fluorescence, $2\times250~\mu\text{L}$ of whole blood or serum was injected into a sealed glass cuvette prefilled with a registered reagent (BioAnalyt 14 iExTM MILA) comprising a mixture of alcohols and organic solvents. The vial was shaken vigorously for 10 seconds and left to settle for 5 minutes in dark conditions to allow separation of solvent and blood. The outer surface of the cuvette was cleaned with a tissue before it was inserted into the machine. Four readings were taken at 20 sec intervals, each time turning the vial by one-quarter. The machine automatically reports the result as the average of these four readings. For each sample, we used the average of two measurements at a one-minute interval (hence 2×4 readings). Data obtained from whole blood was adjusted for hematocrit value, which was estimated from

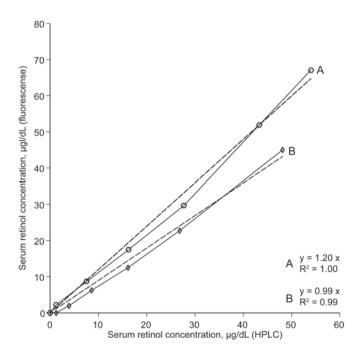


Figure 1: Linearity of retinol concentrations measured by fluorescence and HPLC in μg/dL over a dilution range of whole blood from two individuals (A and B)

hemoglobin levels (Hemocue 301, Ängelholm, Sweden) according to Bioanalyt iCheck guidelines. Standard control samples provided by the manufacturer were measured at the beginning and at the end of each batch of measurements and were within range.

Linearity of the relationship between serum retinol concentrations measured by fluorescence and HPLC was assessed in duplicate over a range of concentrations (100%, 70%, 50%, 35%, 20% and 0 %) in whole blood samples from two individuals diluted with phosphate-buffered saline. The prefilled cuvette used for fluorescence requires a 500 μL sample volume. To assess whether a 250 μL sample would provide similar results as a 500 μL sample we measured blood samples from two individuals as 500 μL whole blood, 500 μL serum and 250 μL serum + 250 μL phosphate-buffered saline in duplicate and compared it to HPLC concentrations. To correct for the lower serum volume in the latter we multiplied the results by two.

Retinol concentrations measured by fluorescence could potentially be influenced by storage time, temperature of the environment, and light, which are typical factors that can vary in field settings. To assess the effect of each of these factors, we first stored whole blood samples of 3 individuals in triplicate for 30 minutes, 1 hour, 7 hours and 24 hours at 4°C in dark conditions, followed by fluorescence measurement. Secondly, we exposed 6 samples with varying concentrations to increasing temperatures (15 °C, 20 °C, 25 °C, 30 °C and 35 °C) in a climate room with a constant humidity of 50%-60% and artificial light. Samples and machine were allowed to adapt to the indicated temperatures for 10 minutes, starting with 15 °C.



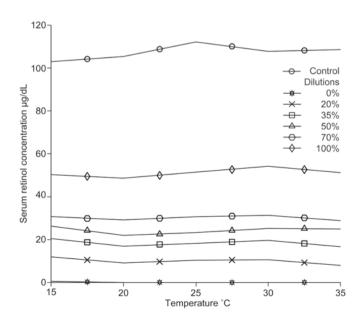


Figure 2: Retinol concentrations measured by fluorescence over a temperature range of 15° to 30° in 6 different dilutions

Lastly, we exposed 6 samples with varying concentrations to three different light conditions:

- 1. in the laboratory, at 4 meters removed from the window with artificial room lights on
- 2. outside at mid-morning with major overcast conditions and no visible sun
- 3. in the laboratory, close to the window with artificial lights on

Findings and conclusion

Linearity of retinol concentration measured by fluorescence and HPLC from two individuals was good, with high correlation and good association (Figure 1). We found no meaningful difference between results derived from whole blood samples and those from serum. At 7h and 24h of storage, concentrations were reduced by 3.5% and 12%, respectively. We found no evidence that storage for 1 hour or less influenced retinol concentrations. We found no evidence that temperature affected retinol concentrations measured by fluorescence (Figure 2). We found a clear effect of light conditions: samples measured outside and close to a window gave consistently higher retinol values than those measured inside (Figure 3).

Based on our results we conclude that the iCheck method provides good linearity when compared to retinol by HPLC. Light has a clear influence on the measurements and therefore the method should be used under controlled light conditions.

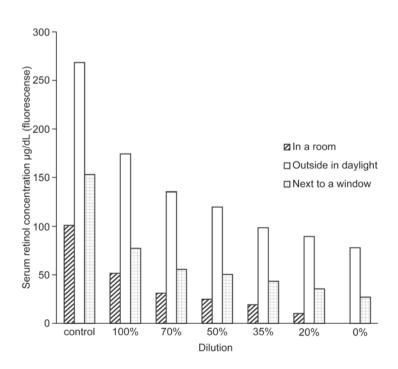


Figure 3: Retinol concentrations measured by fluorescence during three different light conditions

Supplement 2

Paired values for sensitivity and specificity of a diagnostic test that provide unbiased estimates of the prevalence of vitamin A deficiency

Let true vitamin A status be defined by the reference method, and let it be indicated by a diagnostic test with a binary outcome. Table 1 shows the cross-tabulated data that might be obtained in a survey.

Table 1: Cross tabulated data for true vitamin A status, defined by the reference method, and diagnostic test results

Diagnostic test result	Vitamin A deficient		Total
	Yes	No	
Positive	А	В	Р
Negative	С	D	Q
Total	R	Т	N

Capital letters in each cell indicate the number of people in that cell

The true and estimated prevalence of vitamin A deficiency can thus be noted as follows:

$$Prev_{\tau} = R/N$$

$$Prev_{\scriptscriptstyle F} = P/N$$

We can show that the estimated prevalence is a function of sensitivity, specificity and the true prevalence:



i. Sensitivity = A/R, hence
$$A = R \times Sensitivity$$

ii.
$$(1 - Specificity) = B/T$$
, hence $B = T \times (1 - Specificity)$

thus:
$$Prev_{\varepsilon} = P/N = (A+B)/N =$$

$$[R \times Sensitivity + T \times (1 - Specificity)]/N =$$

$$(R \times Sensitivity)/N + [(N-R) \times (1-Specificity)]/N =$$

$$Prev_{\tau} \times Sensitivity + (1-PrevT) \times (1-Specificity)$$

if $Prev = Prev_{T} = Prev_{E}$ it can be derived that:

Sensitivity =
$$1 + (1 - 1/Prev) \times (1 - Specificity)$$

Note that the association between *Sensitivity* and (1-Specificity) is linear, with Sensitivity = 1 when (1-Specificity) = 0, and slope defined by (1 - 1/Prev). This linear association is show in Figure 4 (main text of article) for different, arbitrarily chosen values of Prev. Thus each line represents an infinite number of paired values for Sensitivity and (1-Specificity), given a true prevalence, that will allow for an unbiased estimation of that prevalence.

Abstract

Background: Biofortified yellow cassava can increase vitamin A intake, but it is unknown how this will affect other nutrient gaps. Our objective was to evaluate whether inclusion of a school lunch with yellow cassava, as compared to either no lunch, or a lunch with maize and beans, can theoretically ensure a nutritionally adequate diet for schoolchildren in Kenya by using the OptiFood linear programming tool.

Methods: Dietary intake of 150 school children aged 7-9 years in Kibwezi district in Eastern Kenya was assessed using a quantitative multi-pass 24-hour recall. Model parameters were derived, including a list of foods consumed, median serving sizes, distribution of frequencies and cost of diet. Food based dietary guidelines were formulated with the linear programming tool for three models: (1) baseline diet comprising exclusively foods not provided at school but mainly at home, (2) baseline diet complemented with a common school lunch of cooked maize and beans, and (3) baseline diet plus a school lunch of cooked yellow cassava. The target for nutrient adequacy was set at 100% of the recommended nutrient intake (RNI) for selecting the best diet, and this was further modeled with promising (nutrient dense) foods to arrive at the nutritionally most optimal and affordable diet.

Results: Out of 13 nutrients, model 3 (yellow cassava) best met the target with adequate intake of 6 nutrients as compared to model 1 (4 nutrients) or model 2 (5 nutrients). However, even in the best scenario with addition of promising foods (i.e. small dried fish and oil) the nutrient adequacy of fat, riboflavin, niacin, folate and vitamin A (range 30-64% of the RNI) could not be ensured.

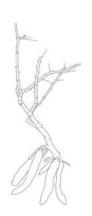
Conclusions: OptiFood is a useful tool to assess the contribution of a biofortified crop to the nutrient adequacy of children. Introduction of yellow cassava should be accompanied by approaches to improve the local diet with fish and oil, and alternative interventions should be formulated to fully eliminate nutrient inadequacy of schoolchildren in Kenya.

Chapter 5

The contribution of yellow cassava to nutrient adequacy of primary school children; the use of linear programming



Elise F. Talsma Karin J. Borgonjen-van den Berg Alida Melse-Boonstra Frans J. Kok Inge D. Brouwer



Introduction

Biofortification of staple foods has the potential to increase the micronutrient intake substantially and is recognized as a sustainable food based approach to reduce micronutrient deficiencies^[1,2,3]. Cassava is a drought resistant crop that does not need fertile soil to grow, can be multiplied by self-propagation and is a staple food for millions of people in Africa^[4]. Biofortified yellow cassava is a new staple food that is richer in provitamin A compared to the common white variety and has been developed through natural plant-breeding techniques^[5,6]. Yellow cassava could potentially reduce vitamin A deficiency especially in rural and remote areas where other interventions cannot reach the people in need of vitamin A^[7,8].

Although yellow cassava is an excellent source for β -carotene and energy, it is known to be generally poor in other nutrients such as iron and zinc^[9]. Children whose diets consist largely of cassava may be vulnerable to micronutrient deficiencies^[10]. As diets in resource poor environments often lack multiple micronutrients^[11], the introduction of yellow cassava, even when only replacement of white cassava is targeted, may require additional food-based dietary adaptations to fill the existing gap in nutrients in the diet.

OptiFood, a software program based on linear programming, enables the development of population-specific food-based recommendations based on current dietary practices and costs^[12]. As the developed guidelines resemble the local diet as close as possible, they are more likely to be followed than more general guidelines that may deviate from local habits. The mathematical modeling of nutrient intake data provides an objective approach to predict, for example, the potential of fortification, supplementation and complementary food products^[13,14,15,16] for fulfilling nutrient needs of infant and young children, and pregnant and lactating women. To our knowledge, OptiFood has not yet been used to develop additional dietary guidelines when introducing a biofortified crop with elevated levels of a single nutrient.

School-age children are a neglected group in terms of micronutrient interventions as they are normally not reached by the usual intervention strategies targeting preschool children or pregnant women^[17]. School-feeding programs often focus on relieving short-term hunger and do not always focus on reducing or preventing micronutrient deficiencies. In Kenya, a commonly provided school lunch comprises a mix of cooked maize and beans^[18]. Adding yellow cassava, currently grown experimentally in Kenya, to the school feeding program could improve micronutrient intake of schoolchildren but there may be need for additional food based dietary guidelines. Based on dietary intake data collected within a larger research project on efficacy of yellow cassava to increase vitamin A status, this paper used OptiFood to evaluate whether inclusion of a yellow cassava in school lunch, as compared to no lunch, or a lunch with maize and beans, can theoretically ensure a nutritionally adequate diet for schoolchildren in Kenya.

Materials and methods

Study design

This study was part of a randomized controlled trial on the efficacy of yellow cassava in improving vitamin A status, carried out from May to November 2012 in Kibwezi district, Eastern province, Kenya. The trial consisted of 3 intervention groups, two provided with a daily ration of white cassava and one group with yellow cassava (this thesis, chapter 2). Dietary intake data was collected during this intervention trial to quantify food intake during and outside school time of all children. This data was used to identify diets with the best possible micronutrient adequacy using linear programming. Three diets were modeled:

- 1. Baseline diet without school lunch
- 2. Addition of a standard school lunch with cooked maize and beans
- 3. Addition of a school lunch of cooked vellow cassava

The trial, including the dietary intake study described here, was approved by ethical committees in Kenya and the Netherlands and registered at clinicaltrials. gov with identifier NCT01614483. Written informed consent of participation in this trial was obtained from parents and children.

Subjects

The intervention trial involved 342 children (5-13 years old) in three primary schools. During screening, children were assessed and ranked according to serum retinol binding protein concentration and those at the lowest end of the distribution were selected for the subsequent trial (this thesis, chapter 2). Children between 7 and 9 years of age represented the largest group in the intervention trial. Therefore all children in this age group involved in the trial (n=150) were selected for this modeling exercise.



Data collection by 24 hour recall

Dietary intake of the subjects was assessed using a quantitative multi-pass 24hour recall[19,20,21] repeated in a subsample (n=44) on a non-consecutive day with all days evenly distributed over the week. The study was carried out by welltrained interviewers in week 13-16 of the intervention in October 2012. Primary caretakers in the presence of the child were asked at home to name all the foods and drinks consumed in and outside the home (except the school lunch) by their child during the preceding day and to describe ingredients and cooking methods of any mixed dishes. Duplicate amounts of all foods, beverages, ingredients of mixed dishes consumed were weighed to the nearest 2 gram using Soehnle electronic kitchen scale (Plateau Art 65086, Germany). When not available, amounts were estimated either in household units, in volume, or in monetary value. The total volume of food cooked at the respondents' household and the volume of food specifically consumed by the child were measured to determine proportion consumed. This proportion was multiplied by the total amount of ingredients used in the preparation of the dish to determine amount of ingredients consumed by the child. Standard recipes were generated to take care of all foods consumed outside the home. Weekly frequencies of consumption of foods/ingredients were asked. Conversion factors from household units, volume and monetary values to weight equivalent were determined. Each type of food consumed was bought at three different places to calculate the mean price per 100 g edible food.

Data preparation and analysis for OptiFood

Data preparation

24 hour recall data were used to define the model parameters using the nutrient calculation system Compleat (version 1.0, Wageningen University, the Netherlands), Excel 2010 (Microsoft Corporation), IBM SPSS (v21) and MS Access 2010. Model parameters include a list of foods consumed by ≥5% of the children. The serving size of each food was defined as the median serving size for all children who consumed the respective food. The minimum number of servings per food, food group and sub-food group was set to 0. The frequency in the 95th percentile was defined as maximum number of servings per food group and sub-food group. The maximum number of servings per single food within a subgroup was estimated based on percentage of children consuming that food. For the school lunch with yellow cassava and with maize and beans, the maximum number of servings per week was five, based on the assumption of providing these foods as school lunch five days a week. The weekly cost of diet was calculated based on the mean daily cost of all 24-h recalls multiplied by seven. Energy constraints were estimated by using the mean body weights and energy requirements for this target group^[22]. The FAO/WHO recommended nutrient intakes (RNIs) are used with 18 mg/day as RNI for iron^[23] (assuming 5% bioavailability), and 5 mg/day for zinc^[24] (assuming 30% bioavailability).

Nutrient intake calculations were based on a food composition table developed specifically for this study, using the national food composition table of Kenya^[25] as primary source complemented with data from food composition tables from South Africa^[26], Mali^[27], East Africa^[28], International Minilist^[29] and United States Department of Agriculture database^[30]. USDA retention factors release 6^[31] were applied to raw ingredients and foods to account for nutrient losses during food preparation. β -carotene and retinol were converted into retinol activity equivalent (RAE) using the International Vitamin A Consultative Group recommended conversion factors^[32,33]. Total β -carotene concentration of boiled yellow and white cassava was used as analyzed (described below). Conversion factors for β -carotene in cassava were conservatively assumed to be 7:1, based on a study in healthy Americans with yellow cassava^[34].

OptiFood analysis

All analyses were carried out with OptiFood (V4.0.4.0), a 4-module-approach, based on linear programming to design population-specific food-based recommendations [12,15]. Three diets were modeled: (1) baseline diet comprising exclusively foods not provided at school but mainly at home, (2) baseline diet complemented with a meal of cooked maize and beans, a commonly provided school lunch, and (3) baseline diet complemented with a school lunch of cooked yellow cassava. In module 1 the model parameters for realistic guidelines including actual dietary patterns, reference values for recommended nutrient intakes and cost information about each food, were checked. In module 2, the nutritionally best diet was identified with and without taking the usual standard food pattern into account. The standard food pattern modeling approach represents the usual food pattern based on the median frequencies of foods consumed. The non-standard food pattern deviates from the median frequencies while remaining within the minimum and maximum limits of number of servings. Based on module 2 the diet that came as

close as possible to meeting nutrient needs was selected for subsequent analysis. In module 3 the robustness of the food-based dietary guidelines of the selected diet was evaluated by identifying the lowest (worst scenario) and highest (best scenario) achievable level for each nutrient. The worst scenario includes the low nutrient dense foods per food group to verify the lowest possible nutrient intake within the modeled diet. The best scenario includes the high nutrient dense foods of each food group to verify the highest possible nutrient intake within the modeled diet. Major problem nutrients were defined as nutrients that did not achieve 100% of their RNI requirements in the best scenario. Nutrients below the 60% of their requirements in the worst scenario were defined as partial problem nutrients. In module 4 promising foods that contributed more than 20% to the intake of problem nutrients were modeled into the diet to evaluate whether inclusion would approach nutrient adequacy closer to the 100% RNI.

Other data collection and analyses

Foods consumed at school

Both white and yellow cassava was grown by the Kenya Agriculture Research Institute (KARI) at Kiboko farm, Kenya and a mixture of different yellow varieties was used. During the trial, cassava was served as a midmorning snack of boiled white or yellow cassava at a portion size of 325 g or 375 g fresh weight for children aged 5-8 year and 9-13 years, respectively. The study also provided a school lunch made of a mix of cooked maize and beans. The exact amount of cassava consumed was recorded by the difference in the weight of the plate of each child before and after eating. A portion of 375 g of the cooked white and yellow cassava was sampled daily at each school and mixed with a food processor after adding 2.5 ml per kg of sample of tert-butylhydroguinone in methanol solution (20 g/100 ml) to prevent antioxidant activity in the food during storage. After mixing, duplicate samples of 15 g per day were pooled by week per school and stored at -15 °C in Kenya, transported on dry ice and stored at -80 °C at the laboratory of the Division of Human Nutrition in Wageningen, the Netherlands and analyzed as described in chapter 2 of this thesis. Concerning maize and beans dish given at schools, the cook was asked to provide three standard portions and the average weight was defined as median portion size. Samples of the maize and beans dish were not analyzed.

Blood samples collection and analysis

All venous blood samples were obtained from fasted children at the start of the trial by venipuncture in a 6 ml trace element tube without additive (BD) and in a 3 ml K2EDTA tube (BD). Samples for retinol analyses were shielded from light and processed under subdued light conditions. All blood samples were stored at 2–8°C until centrifuging (1200 g, 10 minutes) and aliquots of serum were stored in liquid nitrogen (–196°C) in Kenya, and at –80 °C during transport and storage at the laboratory of the Division of Human Nutrition in Wageningen, the Netherlands. Concentrations of serum retinol are analysed at Wageningen University, the Netherlands (May, 2013) as described in detail in chapter 2 of this thesis. Serum concentrations of C-reactive protein, a1-acid glycoprotein, serum ferritin concentration, serum soluble transferrin receptor, serum vitamin B12 and serum zinc concentration were measured at the Meander Medical Hospital, Amersfoort, the Netherlands on a Beckman Coulter UniCel DxC 880i analyzer as per manufacturer's instructions. Hemoglobin was measured using a Celltac-a



automated hematology analyzer (MEK-6410K) in Makindu hospital, Kenya as per manufacturer's instructions within 6 hours after sample collection.

Weight and height

Weight and height were measured at baseline according to WHO guidelines^[35] to the nearest 0.1 kg and 0.1 cm using a mechanical floor scale and a portable stadiometer (Seca, Hamburg, Germany). Anthropometric indices were calculated by ANTHRO-plus (WHOv3.2.2, www.who.int/childgrowth/software/en/). Stunting was defined as z-score for height-for-age less than -2 SD.

Results

Baseline characteristics

The children in our study were on average 8.5 years old with 27% being stunted (Table 1). Thirty percent of the children suffered from vitamin A deficiency but almost none were zinc deficient (3%). Vitamin B12 status was in general low with 58% of the children being mild deficient and 37% severe or moderate deficient. Anemia prevalence was only 6% but 26% of the children were iron deficient. Inflammation affected 20% of our population. Level of inflammation has not been taken into account in the above prevalence estimates.

Food intake

In total 194 recalls were used for data analysis irrespective whether a first or a second recall. In total, 20 out of the total of 51 food items were consumed by more than 5% of the children (Table 2). Foods most commonly consumed were maize, both as whole grain and flour, beans, onions and tomatoes with salt and oil. Serving sizes in the baseline diet varied from 0.4 g/day for baking powder to 167 g/day for maize flour. Most foods had serving sizes > 10 g/day (n=14, 70%). White sugar, oil and onion were consumed in small portion sizes (<10 g/day). The price of a daily diet was estimated to be 30 KsH per day.

The contribution of yellow cassava to nutrient adequacy

Table 1: Nutrition status indicators of the study population (n=150)

Characteristic	Mean	SD
Background		
Age, years	8.5	(0.9)
Sex, girls	74	(49%)
Anthropometrics		
Body-mass-index-for-age ¹ z-score, SD	-1.4	(0.9)
Inflammation ⁸ , n	31	(20%)



Characteristic	Mean	SD
Height-for-age ¹ z-score, SD	-1.2	(1.0)
Children being stunted ¹	41	(27%)
Micronutrient markers		
Serum retinol concentration, µmol/L	0.81	(0.18)
Vitamin A deficiency ²	45	(30%)
Serum zinc concentration, µmol/L	13.7	(2.2)
Zinc deficiency ³ , n (%)	5	(3%)
Serum vitamin B12, pmol/L	123	[97,160]
Vitamin B12 mild deficiency ^{4*}	84	(58%)
Vitamin B12 moderate/severe deficiency ^{5*}	54	(37%)
Haemoglobin concentration, g/L	131	(11)
Anaemia ⁶	9	(6%)
Iron deficiency ^{7*}	53	(36%)
Inflammation ⁸ , n	31	(20%)

Values indicate mean (SD), median [25-75th percentile] or n(%) unless indicated otherwise

^{*} n=146 for vitamin B12 and iron deficiency

¹ WHO 2006 reference population

 $^{^2}$ Vitamin A deficiency: Serum retinol concentration < 0.70 μ mol/L.

 $^{^3}$ Zinc deficiency: Serum zinc concentration < 9.9 μ mol/L.

 $^{^4}$ Vitamin B12 mild deficiency: Serum B12 < 133 pmol/L

 $^{^{5}}$ Vitamin B12 moderate/severe deficiency: Serum B12 < 107 pmol/L

 $^{^{6}}$ Anemia is defined by Hemoglobin concentration < 115 g/L for children aged 5 to 11 years

 $^{^{7}}$ Iron deficiency: Serum ferritin concentration <15 $\mu g/L$ and Soluble transferrin receptor >1.55 mg/L

 $^{^8}$ Defined by Serum concentrations of C-reactive protein > 5 mg/L and/or a1-acid glycoprotein > 1 $\mu g/L$.

The contribution of yellow cassava to nutrient adequacy

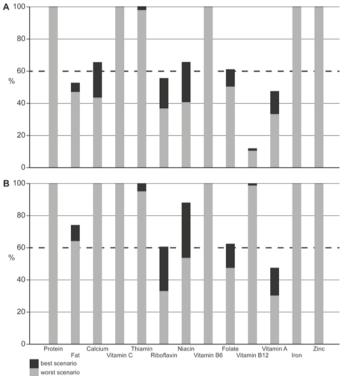


Figure 1: Nutrient intake as percentage of the RNI for yellow cassava diet and optimized diet

A For yellow cassava only

B For yellow cassava with the addition of oil and small dried fish as promising foods



Table 2: Foods consumed by schoolchildren (7-9 years) in Kibwezi district, Kenya

	Consumed by % of children 1	Serving size ² g/day	Min servings ³ per week	Max servings ⁴ per week	Cost ⁵ Ksh/100g
Added fats					
Oil	71	8.7	0	7	20
Added sugars					
Sugar brown	21	15.2	0	4	12
Sugar white	12	9.7	0	3	12
Dairy products					
Milk cow	23	39.4	0	7	8
Grains & grain products					
Flour maize white	67	166.9	0	7	6
Flour wheat refined	16	97.2	0	5	6
Rice refined	7	90.2	0	2	12
Maize grains white, dried	60	123.7	0	6	5
Maize hulled white, dried	5	139.1	0	1	9
Legumes, nuts & seeds					
Beans red, dried	36	38.8	0	6	10
Beans mung, dried	6	49.8	0	1	10
Peas pigeon, dried	24	52.6	0	5	8
Peas cow, dried	8	55.7	0	2	7
Miscellaneous					
Salt, iodized	82	2.8	0	7	3
Bouillon powder	9	1.3	0	7	46
Baking powder	5	0.4	0	7	20
Vegetables					
Onion bulb red	67	4.3	0	7	10
Tomato	62	30.4	0	7	5
Cabbage	31	98.3	0	7	3
Kale/collard greens	24	33.4	0	7	8
Promising foods					
Composites (mixed food grou	ps)				
Maize & beans meal	67	306.0	0	5	0
Starchy roots & other starchy	plant foods				
Yellow cassava ⁶	26	372.0	0	5	0
Meat, fish & eggs					
Small fish, dried	1	89.0	0	7	33

 $^{^{\}mathrm{1}}$ All foods consumed by at least 5% of the children, and small fish

 $^{^{\}rm 2}$ Values are median serving sizes of the raw edible portions based on 24-h recalls

³ Minimum frequencies for all foods were set to 0

⁴ Maximum frequencies were values in the 95th percentile of distribution

⁵ Mean costs in Kenyan Shilling per 100 gram edible portion, estimated from 3 different kind of shops/markets

 $^{^6}$ Contains 394 µg β -carotene per 100 g boiled fresh weight

Table 4: Nutrient composition and diet costs of yellow cassava diet for module 2, 3 and 4

						%	% of Recommended Nutrient Intake	ended Nu	trient In	take						COST
		Energy		Protein Calcium	Fat	Vit. C	Thiamin Riboflavin		Niacin	Vit. B6	Folate	Vit. B12	Vit. A	Iron	Zinc	KSH/day
M2	Yellow cassava diet															
	Non-standard food pattern	100	226	52	26	338	138	47	63	135	27	11	45	122	139	30
M3	Yellow cassava diet															
	Worst scenario	92	213	47	44	236	66	37	41	102	20	11	33	115	104	28
	Best scenario	138	230	535	66 ²	431	145	56 ₂	66 ²	145	612	122	485	122	145	38
⊼	M4 Worst scenario of yellow cassava with promising foods	th promisi	ng foods													
	1 Oil, 7 servings/week	93	205	623	44	234	95	37	40	101	20	10	33	112	102	28
	2 Maize grains, 4 servings/week ¹	98	213	52	48	277	155	53	57	147	22	7	35	118	160	28
	3 Milk, 2.2 servings/week	92	214	48	46	233	86	40	41	102	51	13	34	114	105	29
	4 Red beans, 7 servings/week	94	238	45	45	234	101	39	43	107	65 ₃	00	32	124	120	29
	5 Sukuma wiki, 7 servings/week	94	219	46	59	351	118	35	26	122	51	7	26	120	114	31
	6 Small dried fish, 1.5 servings/ week	63	237	20	1103	230	67	33	54	108	48	666	30	115	119	30
	1+4+6 (oil + red beans + fish)	91	225	64	101	230	82	30	48	106	47	66	30	96	109	30
	$1+6 (oil + fish)^4$	94	228	64	110	230	95	33	54	108	48	66	30	112	118	30

Values are expressed as percentage of recommended nutrient intakes (RNI)

¹ 7 servings/week extra grain and grain products of which 4 serving/week maize grains

 2 Major problem nutrients that did not achieve 100% of their RNI requirements in the best scenario

³ Nutrient values increased to >60% of the RNI by adding the particular food to the food based dietary guidelines

⁴ Best optimized diet



Table 3: Maximized nutrient composition (%RNI) of three diets using the standard or the non-standard food pattern for schoolchildren in Kibwezi district, Kenya

	'					
	No Scho	ol Lunch	Maize and bear	ns school lunch	Yellow cassav	a school lunch
	Standard food pattern	Non-standard food pattern	Standard food pattern	Non-standard food pattern	Standard food pattern	Non-standard food pattern
Protein	217	222	216	251	194	226
Fat	44	44	49	46	55	52
Calcium	33	37	38	49	44	56
Vitamin C	100	100	199	147	427	338
Thiamin	101	107	138	130	148	138
Riboflavin	31	29	49	59	38	47
Niacin	45	50	59	68	54	63
Vitamin B6	51	55	83	85	135	135
Folate	34	33	43	62	43	57
Vitamin B12	4	3	6	11	6	11
Vitamin A	4	13	15	16	45	45
Iron	86	88	97	101	115	122
Zinc	115	117	133	152	123	139
Cost/Day (Ksh)	30	30	29	30	30	30

All values: % of the Recommended Nutrient Intake (RNI)

Linear modeling

In module 2, fat, calcium, riboflavin, niacin, folate, vitamin B12, and vitamin A intake were below 100% RNI coverage for all three diets (Table 3). Vitamin B6 and iron intake were below 100% RNI in the baseline diet and the maize and beans diet. Only protein, zinc, vitamin C and thiamin intake were above 100% RNI in any of the three diets. The average cost of the modeled diets was 30 KsH. The non-standard food pattern diet showed a better coverage of RNI than the standard food pattern. On average RNI coverage was best in the non-standard food pattern of the yellow cassava diet (6 out of 13 nutrients > 100% RNI) and this diet was used for further modeling in OptiFood. A draft set of food-based dietary quidelines was developed in which RNI coverage in best and worst scenarios was estimated by the extremes of each nutrient intake distribution (Table 4). Fat, calcium, riboflavin, niacin, folate, vitamin B12 and vitamin A were defined as major problem nutrients and none of the nutrients was defined as partial problem nutrient. The promising food items that contributed ≥ 20% to the intake of the problem nutrients (Table 5) were oil for fat, maize grains and milk for riboflavin, maize grains for niacin, red beans for folate, milk for vitamin B12 and kale/collard greens for vitamin A. Small dried fish was only consumed by one child but was identified as a promising food to contribute to vitamin B12 and calcium intake. A draft set of food based dietary guidelines was developed and presented as worst and best scenario (Table 4). All six promising foods were modeled individually into the yellow cassava diet and a new set of food based dietary guidelines was developed. Oil, red beans and small dried fish increased the intake of at least one nutrient to >60% of the RNI in the worst scenario and were combined to produce the final alternative sets. In addition to oil and small dried fish, red beans did not provide any improvement on the nutrient intake but decreased the intake of nearly all nutrients. Therefore oil and small dried fish as most promising foods were added to the yellow cassava diet to represent the optimal modeled diet.

Final food based dietary guidelines

The final food based dietary guideline for the optimized diet contained 14 weekly servings of oil, no dairy products, 13.2 servings of grain products, 13 servings of legumes, nuts or seeds, 1.5 serving of small dried fish, 5 servings of yellow cassava (provided as school lunch) and 0.8 serving of vegetables (Table 6). In the optimized diet calcium and vitamin B12 were no longer problem nutrients; however fat, riboflavin, niacin, folate, and vitamin A did not reach 100% RNI in the best scenario (range 30-64%) and remained problem nutrients (Figure 1).

Table 5: Contribution of the main food sources to the intake of major problem nutrients

Major problem nutrient	Promising food	Contribution to intake (%)
Fat	Oil	31
Calcium	Pigeon peas	19
	Small dried fish ¹	
Riboflavin	Maize grains	28
	Milk	20
Niacin	Maize grains	28
Folate	Red beans	23
Vitamin B12	Milk	65
	Small dried fish ¹	
Vitamin A	Kale/collard greens	26

 $^{^{\}scriptscriptstyle 1}$ Small fish was not included in module 1 and 2 as only 1 child consumed small fish

Table 6: Recommended servings per week by food group in yellow cassava diet and best optimized diet

	Cassava diet	Optimized diet
		servings / week
Added fats	7	14
Dairy products	7	0
Grains & grain products	13.8	13.2
Legumes, nuts & seeds	14	13
Meat, fish & eggs		1.5
of which small dried fish		1.5
Starchy roots & other starchy plant foods	5	5
of which yellow cassava	5	5
Vegetables	9.5	0.8





Discussion

We found that yellow cassava provided as a school lunch has the potential to improve the nutrient adequacy of the diet and helps to better ensure dietary quality compared to no school lunch or a school lunch with maize and beans. However, even with the addition of promising foods (small dried fish and oil) the adequacy of fat, riboflavin, niacin, folate and vitamin A intake in the nutritionally best diets could not be ensured when complying to the used energy and costs constraints.

The study shows that the vitamin A intake through the standard diet was low (20 μg RAE/day, see chapter 2) and covered only 3-4% of the RNI. This is also reflected in the high prevalence of vitamin A deficiency of 30%, considered as being of severe public health concern^[36]. A study conducted in another rural part of Kenya found a much higher vitamin A intake among schoolchildren, probably due to their intervention in which vitamin A enriched oil was provided[37]. One of the main food sources of vitamin A in our population was Collard greens/Kale (Sukuma Wiki). This is a commonly and frequently consumed green leafy vegetable but was consumed in rather small amounts (~30 grams) by our children. Further, retention of B-carotene after cooking is likely to be low since it is commonly boiled for a long time[38]. Also, vitamin A bioavailability of green leafy vegetables is known to be low^[39,40,41] In addition, children selected for the present study represented the lowest end of distribution of vitamin A status in the area. Therefore, diets of our children used for modeling may also represent the lowest extreme with regard to vitamin A intake, which may explain why their requirement could not been attained. Lastly, our study was carried out during the dry season, reflecting a limited availability of foods[42].

Remarkably, the modeled optimal diet including yellow cassava would only ensure about 45% of the required vitamin A intake and this was not meaningfully improved through addition of promising foods. Another study using a modelling approach to estimate vitamin A intake in 6 countries in Africa (Kenya not included) found that replacement of white by yellow cassava would provide an adequate vitamin A intake[8]. However, differences in assumptions as compared to our study may explain the divergence; first their model was based on a variety of yellow cassava with β -carotene content of 21 $\mu g/g$, a high concentration currently not available in Africa. The only yellow cassava varieties released to date are in Nigeria with total β -carotene concentrations of approximately 6 μ g/g^[7]. We used β -carotene varieties with an average concentration of 5.4 µg/g (fresh weight). Second, in contrast to Katz's study where retention of β-carotene of 96% was assumed, we have used a more conservative estimate of 73% resulting in β-carotene concentration of 3.9 µg/g (fresh weight). The retention factor used by Katz was based on simmering cassava at low temperature which prevents carotenoid destruction but is not common practice. We based our estimate on boiling the yellow cassava for over an hour on wood fire. In general, retention levels for preparation of yellow cassava ranges from 10% for heavily processed and roasted cassava granules[43] to 87% for boiling^[44]. Third, the conversion factor for β -carotene to retinol in Katz et al. was based on a study with 10 healthy Americans in which de overall conversion factor was 4.5 but with a high variability between subject (0.3-10.6: 1)[34]. We used a conservative conversion factor of 7:1. Our data provides therefore a conservative picture of the potential increases in vitamin A intake to be reached by consuming yellow cassava. The breeding process of yellow cassava is ongoing and varieties with higher β-carotene concentrations are scheduled to be released over the next years and would therefore have a larger impact on vitamin A intake^[45].

In our population, iron and zinc were not identified as a problem nutrient as shown by only 3% of children being zinc deficient in our study population. Although 36% of the children had iron deficiency, only 6% had anemia and iron deficiency was mostly sub-clinical. Major food sources of iron were pigeon peas and maize and although the lowest bioavailability of 5% was applied to these foods, the actual bioavailability may even have been lower^[46,47]. Based on food consumption studies among children 2-5 years old in Kenya and Nigeria, Gegios et al (2010) concluded that children consuming cassava as staple food are at risk for inadequate intake of zinc and iron^[10]. However, in our population cassava was not a staple food and other food sources ensured the adequacy of intake of zinc and iron not affected by the introduction of yellow cassava.

The yellow cassava school lunch complemented with oil and small fish ensured adequate dietary intakes of calcium. This was mainly due to the inclusion of small fish. Whole small fish including edible bones has proven to be a good source of calcium with a bioavailability comparable to that of milk. However, in our population only one child had consumed small fish. The reason why schoolchildren do not consume small fish or fish in general is unknown. Since the research site was not in an aquatic environment, availability could be a constraint to consumption as well as the general fear of children to have a bone struck in their throat.

Cost may be a constraint to consuming nutrient dense foods, but running a cost analyses of the optimal diet shows still a nutrient content below 100% RNI for fat (88% RNI) riboflavin (79% RNI), folate (62% RNI) and vitamin A (48% RNI) by a price of 60 Ksh per day. The low diversity of the foods in the monotonous diet (only 20 different food items are consumed by more than 5% of the children), the low frequency and small quantity of foods rich in nutrients consumed is a major limiting factor. However, addition of nutrient dense food that is not or hardly consumed would probably also not provide realistic options.

The food based dietary guidelines in the best scenario covered over 200% of the protein and vitamin C requirements and over 100% of vitamin B6, iron and zinc requirements. These intakes were still below the upper level of recommended intakes and therefore will do no harm^[23,24,33]. We did not consider the quality of the protein consumed. However, the biological value of the protein may be low given that cereals contributed most to protein intake being limited in growth supporting lysine. The digestibility of the protein may also be compromised given the high concentration of dietary fiber in the meals^[48].

Limitations of the dietary data used as input for the OptiFood analysis are related to weaknesses inherent to the 24-hour recall. To reduce bias, we took precautions through training of interviewers, impromptu supervision to minimize reporting errors, proper calibration of instruments and random assignment of interviewers. Nevertheless some errors may have inevitably occurred in recalling the dietary intake. To minimize misreporting of food intake, caregivers were taken through a systematic multiple-pass procedure which facilitated recollection of food and ingredients used in preparation of meals at home^[19]. Also, because mothers were likely to omit foods consumed out-of-home such as fruits and snacks^[49], children were present to assist with foods that were consumed out of home. The use of household measures from the respondent's own home aided them to remember and to estimate quantities and portion sizes, and when food ingredients were available actual weights of a duplicate portion were taken to avoid mistakes in estimations. The use of food consumption tables to convert food intake into nutrients may have





introduced bias into nutrient intake estimates [50]. The Kenyan food composition table [25] was the primary source of nutrient composition supplemented with values from other data bases for foods and nutrients not available in the primary source following international standards for food composition and compilation [51]. Despite these limitations, the main contributor to β -carotene intake in our study was the yellow cassava, which was analyzed in our laboratory as cooked cassava to be able to quantify the exact exposure per day.

We assumed that the foods consumed out-of-school in our population reflected the habitual diet of schoolchildren implicitly anticipating that children in general do not take food to school and that food provided in school would not affect the amount of food consumed at home. A study in rural Kenya did not find evidence that schoolchildren who received supplementary snacks at school experienced reduced intakes at home^[52]. However, an earlier study in the same area and season among the same age group indicated that some children were used to take a meal (consisting of maize and beans) to school. Our assumption may have therefore led to a slight underestimation of the OptiFood model parameters related to portion size and frequency of consumption, especially concerning maize and beans. As these foods are not major contributors to the nutrients identified as being in short supply, we assume a negligible effect on the modeling results.

Our study shows that providing yellow cassava in a school feeding program can have a major impact on the nutrient adequacy of the diet in our study area. However, additional measures would still be needed to fully eliminate nutrient inadequacy. To keep such measures as close as possible to the existing diet, increasing the consumption of small fish and oil would be the best achievable options to further improve nutrient adequacy. Consultation with local stakeholders should reveal whether these adaptations to the dietary behavior are realistic and feasible^[16]. Furthermore, deficits of multiple micronutrients such as vitamin A. folate and riboflavin will remain. Since the extent of the multiple micronutrient deficits in the modeled diets would be difficult to meet with a single intervention product, introduction of yellow cassava in this area should be integrated with approaches that encourage changes to the traditional diets as well as introduction of improved products such as fortified foods or improved nutrient supplements. Our study also showed that linear modeling of dietary intake data provides an excellent tool to evaluate and develop nutritional strategies for achieving better nutritional adequacy under local circumstances.

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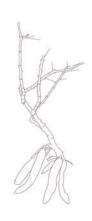




Chapter 6



General Discussion



The overall aim of this thesis is to provide proof of principle whether biofortified yellow cassava can improve the vitamin A status of marginally deficient children in Kenya. This chapter summarizes the main findings of the (research and discusses outstanding methodological issues and key findings in a broader context, followed by suggestions for future research and discussion of the main implications of our findings for public health in low- and middle- income populations.

The main findings are displayed in table 1. In summary, we found that daily consumption of yellow cassava increased serum retinol concentration, and especially serum β-carotene concentration (chapter 2), of marginal vitamin A deficient primary school children in Kenya. We assessed acceptability, both sensory and culturally, and found that caretakers as well as children preferred yellow over white cassava because of its soft texture, sweet taste and attractive color (chapter 3). Almost all caretakers had the intention to prepare yellow cassava for their children. To be able to determine vitamin A deficiency in a low resource setting, we found that a combination of proxy markers; retinol binding protein, transthyretin and C-reactive protein, provided the best estimation in comparison with serum retinol by HPLC (chapter 4). These markers can be combined to estimate the prevalence of vitamin A deficiency in population surveys, based on a simple decision rule to determine individual vitamin A status. Lastly we assessed the nutrient contribution of yellow cassava added to the habitual diet using linear modeling (chapter 5). We found that yellow cassava as a school lunch has the potential to improve the nutrient adequacy of the diet, compared to no school lunch or a school lunch with maize and beans. However, nutrient adequacy could not be ensured for some nutrients and additional dietary guidelines and interventions are needed to fill these gaps.

Table 1: Main findings

Design	Objectives	Main results
Type: RCT Population: Children 5-13 yr in 3 schools Chapter 2	- Efficacy yellow cassava on serum retinol concentration	 Δ Serum retinol difference 0.04 µmol/L (0.00-0.07) β-carotene concentrations increased 524% (448%, 608%)
Type: cross-sectional Population: Children 7-12 yr in 3 schools <i>Chapter 3</i>	- Sensory and culturally accepta- bility of yellow cassava	 72% preferred yellow cassava children 99% had intention to prepare yellow cassava for their children 64% had intention to prepare yellow cassava more than twice per week
Type: cross-sectional Population: Children 6-12 yr in 15 schools Chapter 4	Validate new diagnostic methods to measure vitamin A deficiency in the field Estimate prevalence of vitamin A deficiency in popu- lation	- ROC: AUC of Transthyretin + Retinol binding protein + C-reactive protein = 0.98 - Decision rule to estimate vitamin A deficiency prevalence: {(-15.277 × [RBP, μmol/L] - 7.013×[Transthyretin, μmol/L] + 0.367×[C-reactive protein, mg/L] + 24.714) > -0.237} - 18% vitamin A deficiency prevalence (HPLC)
Type: cross-sectional Population: Children 5-13 yr in 3 schools <i>Chapter 5</i>	Model with linear programming the nutrition adequacy of 1) no school lunch, 2) maize and beans school lunch and 3) yellow cassava lunch	 Yellow cassava provided the best % of the RNI (6 out of 13 nutrients >100% RNI) Promising foods were oil and small dried fish The nutrient adequacy for fat, riboflavin, niacin, folate and vitamin A remained low (30-64%)

(RCT = randomized controlled trial, ROC = Receiver operating characteristic, AUC = Area under de curve, RNI = Recommended nutrient intake)

Internal validity

The methodological issues and key findings of the research described in this thesis have been discussed in the various chapters. Those relevant for the internal validity of the findings are described and mainly relate to selection bias, information bias, and confounding.

Selection bias

Selection bias occurs when the study population does not represent the target population. We purposively selected a target population with low vitamin A status through primary schools. One could speculate that children from poorer households are less likely to go to school and, as poverty is related to malnutrition, are more likely to be vitamin A deficient. By sampling through schools, we may have missed these children in our study population and this might explain that we did not find severe cases of vitamin A deficiency. However, the primary school enrollment rate in Kenya is around 84%^[1] and the related selection bias is assumed to be negligible. In addition, as multiple micronutrient deficiencies often occur simultaneously^[2], the selected children might have been also affected by other micronutrient deficiencies that could have influenced the intervention effect. We have assessed the presence of some, such as iron and zinc deficiency, and controlled for these in the analyses.



Bias due to non-response might have occurred in the efficacy trial described in chapter 2, as out of the initial 1256 children eligible, 80 children were non-responders because they either did not show up at the blood sampling or caretakers refused to provide consent. We do not have information on the characteristics of these non-responders. However, the non-responders reflect only 6% of the original sampling population and therefore we assume the non-response bias to be negligible. Furthermore, we only lost four children to follow up during the intervention, all because they moved out of the research area. Compliance throughout the study was high at 100% and results from data analysis with inclusion of two children that did not comply with the 80% compliance rule (intention to treat analysis) did not differ as compared to per protocol analysis.

In the two cross sectional studies we can also rule out any effect of non-response bias, as all eligible caretakers and children that were selected also participated in the study. Three children in the study on indicators of vitamin A status (chapter 4) were excluded because we were unable to obtain blood samples from them due to small veins. Also here we conclude that if there would potentially be an effect from the non-responders, it is small and it will not change the conclusions drawn in that chapter.

Information or measurement bias

Non-blinding of the treatment in a randomized trial can lead to information bias by the research team as well as the participants. In the efficacy trial in chapter 2, blinding to cassava type was not possible because of the yellow color of cassava. Knowledge of treatment allocation may have affected the compliance and retention of study participants^[3]. However in our study we assume the effects of nonblinding to be small, as both white and yellow cassava were consumed in equal amounts and vitamin A intake from the general diet was comparable between groups. Also the retention of the children was similar in the three intervention groups. To minimize the potential bias, children as well as the research team were

blinded to type of supplement provided until the trial was completed, and the code was only revealed after the statistical analyses plan was finalized. Furthermore, all biochemical analyses were done while being unaware of the treatment allocation.

In chapter 2 and 4, vitamin A deficiency was defined based on serum retinol concentration measured by HPLC as the outcome measure. Although serum retinol concentration is the reference indicator for assessing vitamin A status [4,5], it is under homeostatic control of the liver and does not reflect hepatic vitamin A stores anymore at higher concentrations^[6]. This effect is expected to be seen at a serum retinol concentration of >1.05 µmol/L, whereas linearity of retinol with liver pools is seen in the deficient range from 0.3 - 0.9 µmol/L. The children in our study were clearly in the mild deficient range (~0.80 umol/L). Little is known about inter-individual differences in the exact point where serum retinol concentration starts to curve, and it may be that the homeostatic effect in our study population already occurred at lower serum concentrations. Other methods that we could have used to assess intervention effect are isotopic dilution methods to estimate body retinol pool size[7,8], but such methods require specific equipment that was not available to us at the start of the trial. However, to our opinion serum retinol concentration was a valid indicator in our studies because the children were in the deficient vitamin A status range.

Limitations of the dietary data, used in the efficacy trial in chapter 2 to measure exposure and in chapter 5 as input for the OptiFood analysis, are related to weaknesses inherent to the 24-hour recall. Despite precautions through training of interviewers, impromptu supervision to minimize reporting errors, proper calibration of instruments, random assignment of interviewers to reduce bias, some errors may have inevitably occurred in recalling dietary intake. To minimize misreporting of food intake, caregivers were taken through a systematic multiple-pass procedure which facilitated recollection of food and ingredients used in preparation of meals at home^[9]. Also, because mothers were likely to omit foods consumed out-of-home such as fruits and snacks^[10], children were present to assist with foods that were consumed out of home. The use of household measures from the respondent's own home aided them to remember and to estimate quantities and portion sizes, and when food ingredients were available actual weights of a duplicate portion were taken to avoid mistakes in estimations. The use of food consumption tables to convert food intake into nutrients could introduce bias into nutrient intake estimates[11]. The Kenyan food composition table[12] was the primary source of nutrient composition supplemented with values from other data bases for foods and nutrients not available in the primary source following international standards for food data base development and compilation^[13]. Despite these limitations, the main contributor to β -carotene intake in our study was the yellow cassava, which was analyzed in our laboratory as cooked cassava to be able to quantify the exact exposure per day.

We used a conservative factor of 7:1 for the conversion of β -carotene into retinol in chapter 2 and 5, which is higher than recommended by the Institute of Medicine (12:1 for mixed diets), but lower than was found in a study with yellow cassava in gerbils $(3.7:1)^{[14]}$ and in humans $(4.2:1)^{[15]}$. The study in humans showed a very large variation (range 0.3 to 10.6), and was calculated from a single exposure which might overestimate bioavailability^[16,17]. Moreover, it was conducted in a western setting among adults and therefore we opted for the conservative conversion factor of 7:1. Using the 7:1 conversion factor for β -carotene in retinol

resulted in an intake close to 50% of the estimated average requirement while using the factor 4:1 would bring vitamin A intake at 100% of the estimated requirement level for both the β -carotene supplement group as well as the yellow cassava group. Considering the number of children that were still vitamin A deficient in these two intervention groups at the end of the study, we concluded that a conservative conversion factor is more appropriate and that 4:1 would have overestimated the exposure of the children.

In chapter 5 we assumed that the foods consumed out-of-school in our population reflected the habitual diet of schoolchildren implicitly anticipating that children in general do not take food to school and that food provided in school would not affect the amount of food consumed at home. A study in rural Kenya did not find evidence that schoolchildren who received supplementary snacks at school experienced reduced intakes at home^[18]. However, an earlier study in the same area and season among the same age group indicated that some children were used to take a meal (consisting of maize and beans) to school. Our assumption might therefore have led to a slight underestimation of the OptiFood model parameters related to portion size and frequency of consumption, especially concerning maize and beans. As these foods are not major contributors to the nutrients identified as being in short supply, we assume a negligible effect on the modeling results.



Serum retinol concentration measured by HPLC was the main outcome indicator in chapter 2 and was also used in chapter 4 to assess vitamin A status in comparison with other diagnostic markers. Every biochemical analysis has its own variability and validity. Quality control is therefore an important aspect to reduce this kind of bias. For retinol concentration by HPLC we assessed 10% of analyses in duplicate and CV's% were below 5% which is considered as acceptable. Furthermore a low and a high control sample were analyzed in every run to estimate variation between runs and these were also below 5%. The diagnostic tests used in chapter 4 are ELISA assays that have higher variability than HPLC and again quality control indicated that variability was general acceptable with CV<10%.

Confounding

Vitamin A status can be prognostic for treatment outcome^[19], and to reduce risk of confounding by initial vitamin A status we have used stratified block randomization in the efficacy trial in chapter 2, with stratum corresponding to tertiles of retinol-binding protein concentration. Furthermore, since retinol binding protein concentrations and retinol concentrations are decreased during inflammation^[20], possible interference due to inflammation was reduced as much as possible. For this, firstly, all children were de-wormed two weeks prior to blood sampling to reduce effect of helminthic infections on inflammation; and secondly, children with inflammation or malaria at baseline were excluded from participation. We adjusted the intervention effect for any remaining inflammation by using two types of inflammation markers to be able to identify both early and late convalescence. In addition, we adjusted the intervention effect for baseline vitamin A status and stratified design because of the importance of these factors on the outcome of the intervention.

In the acceptability study in chapter 3, adjustments were made in the regression models to correct for inter-observer variation and for age and education level of the caretaker. Other studies^[21,22,23] using similar models to explain behavior also adjusted the data for these factors and although it only resulted in a slightly

higher explained variance in the regression models, we remained the adjustments in our models in order to be able to compare our results with other research.

External validity

The research described in this thesis was conducted in primary school children, an older age group than the pre-primary school children who have a higher risk of vitamin A deficiency $^{[5,24]}$. Older children were chosen for several reasons. First to reduce possible interference of the national vitamin A supplementation program for children under 5 years of age; second to establish higher cassava and β -carotene intake as older children are able to consume more food; and third, for logistical reasons as the setting within a primary school permitted a higher level of control than home feeding and allowed us to feed large groups of children together. However, our results can be extrapolated to younger children with the notion that we assume that yellow cassava will have a larger impact in the younger age groups since they are more likely to be vitamin A deficient. Especially in areas where vitamin A supplementation programs are poorly implemented, we expect that dissemination of yellow cassava will have impact.

The most important reason for study site selection was vitamin A deficiency prevalence as intervention effect is expected to be higher in vitamin A deficient children. We found an overall prevalence of 18% in 15 primary schools, as described in chapter 4, indicating that vitamin A deficiency is a moderate public health problem as classified by the WHO^[5]. Out of the 15 schools three were selected based on the highest deficiency rates, for participation in the efficacy study described in chapter 2. Within these schools we selected children with the lowest retinol binding protein concentration as a proxy indicator for vitamin A deficiency and prevalence at the start of the trial was 24%. Because these children are more vitamin A deficient than their peers, the intervention effect of yellow cassava might have been larger than it would have been in their less deficient peers. Also, the selected children might have had a lower vitamin A intake, resulting in an underestimation of the adequacy of vitamin A intake that is described in the modeling exercise in chapter 5.

We have conducted the first efficacy trial with biofortified yellow cassava and therefore comparison with other studies has its limitations. The intervention effect of 0.04 µmol/L on serum retinol concentration in the yellow cassava group was modest, but taken together with the large increase in serum β -carotene, it shows that the \(\beta\)-carotene from yellow cassava was well absorbed and that at least some of it was converted to retinol. Some food based trials with provitamin A rich vegetables and fruits have previously shown larger effects on serum retinol concentration and smaller effects on β-carotene concentrations^[25,26]. A study in South African children with a higher dose of \(\beta\)-carotene from biofortified sweet potato (12,375 µg versus 1,463 µg from our yellow cassava) showed an increase in the vitamin A pool, but serum retinol concentration was not evidently increased, despite lower serum retinol concentrations (0.61 µmol/L)[27]. Unfortunately, serum β -carotene was not measured. Another study using biofortified sweet potato in children (with 70% vitamin A deficiency) in Mozambique showed an increase in serum retinol concentration of 0.10 μ mol/L^[28], but also here β -carotene was not measured. In a study in Bangladeshi women, daily consumption of biofortified sweet potato resulted in a large increase in serum β-carotene concentration, but had no evident effect on serum retinol concentration^[29]. In conclusion, some studies show an effect on serum retinol concentrations whereas others do not. However, serum β -carotene concentration increased in all of the three studies mentioned above^[25,26,29]. due to the intervention. Therefore, there may have been factors that have hampered the bioconversion of β -carotene to retinol.

Of all possible factors determining conversion of β -carotene to retinol, the main factor seems to be the converter enzyme BCM01 and its related metabolism. However, subgroup analyses on BCM01 genotypes showed no effect of four BCM01 SNPs genotypes on treatment effect. This might be explained by the SNPs that we used, as they were discovered to have an effect in Caucasian populations and might not play a significant role in Africans. Genetic data on polymorphisms in Africans is lacking in general but the two small African datasets available in the HapMap database are in agreement with our finding that SNP rs7501331 was absent in our population, as it was also virtually absent in populations of Maasai in Kenya and Yoruba in Nigeria 1321. Nonetheless, we cannot exclude the possibility that other polymorphisms in the BCM01 gene occur with high frequencies in our study population, which we have not examined and which produce BCM01 variants with impaired functionality.



A general concern with introducing vitamin A biofortified staple foods is the acceptability of the crop by the community due to a change in appearance and taste^[33]. The introduction of orange fleshed sweet potato was successful, as shown in a study in Mozambique in which the crop was widely consumed by the community^[34]. We introduced yellow cassava in a community where cassava is not unknown, but also not a staple crop. Since previous experience of consumers with a food influences perception and beliefs^[35], a population that consumes white cassava on a daily basis might probably have stronger beliefs towards the attributes of yellow cassava than those consuming cassava less often. We found that consumption of yellow cassava was acceptable in our study site, but whether this counts for other population, especially those consuming cassava as a staple food, remains unknown. However it is promising that a study on yellow cassava in Benin showed that yellow cassava was acceptable to a cassava consuming population^[36].

In chapter 4, we conclude that a combination of three proxy markers performed well in discriminating between children with and without vitamin A deficiency. The search for new, easy to use proxy methods is not new, and retinol binding protein measured by ELISA has also been used by others as a proxy marker[37,38,39]. In general, ELISA assays for measurement of retinol binding protein concentrations are not designed for diagnostic purposes, and measurement results can differ substantially between assays[40] which might be caused by differences in extraction method. This may in part also explain why many different cut-offs have been proposed for retinol protein binding concentrations to indicate vitamin A deficiency[37]. A sandwich ELISA-assay has been developed for simultaneous measurement of retinol binding protein, C-reactive protein, serum ferritin and soluble transferrin receptor and has significantly contributed to the wide scale measurement of micronutrient status in country surveys^[41]. However, this method assumes that retinol and retinol binding protein occurs in a 1:1 ratio in serum and requires continuous calibration against serum retinol measured by HPLC; moreover, there is no certified reference material available for calibration on a wider scale, which limits local use of this assay. In our study we found concentrations of retinol binding protein that were largely below concentrations of serum retinol, which does not correspond with an expected 1:1 ratio. Nevertheless, we observed a remarkable

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goodness of fit of the linear predictor, which deserves further exploration. If our findings can be reproduced in other settings and populations, this would provide a user friendly method that is much easier to conduct locally in comparison to the sandwich ELISA or HPLC.

The food based dietary guidelines developed in chapter 5 are theoretical and require adaptation of the dietary intake habits outside the standard dietary pattern. A study comparable to ours, aiming to identify new guidelines for infant and young child feeding in Guatemala, tested the guidelines after they were formulated with local stakeholders and evaluated feasibility of successful promotion^[42]. We did not perform such a community consultation and evaluations, and therefore acceptability of the developed dietary guidelines to the community or any other population should not be assumed.

Future research

With this thesis, we provide the first evidence that consumption of yellow cassava in marginal vitamin A deficient children in Eastern Kenya, improves serum retinol concentration as well as β -carotene concentration. However, since this is the first randomized controlled study with yellow cassava, our study should be replicated in another population, preferably in younger children, and measurements should include body pool vitamin A in addition to serum retinol concentrations and β -carotene concentration. As we used the preparation method with the highest retention level, priority should be given to a preparation method closer to the local processing habits of a cassava consuming population. To increase efficacy, new varieties of yellow cassava should be used with higher β -carotene concentrations.

The bioefficacy of β -carotene is an important aspect of the efficacy of yellow cassava and should be the focus of further research on genetic differences in conversion ability of β -carotene. DNA sequencing of African populations may reveal new common SNPs in the BCM01 gene as well as SNPs in other metabolic processes of retinoids, for example in fat metabolism or in retinol transporter protein synthesis such as retinol binding protein and transthyretin. Dose response studies with β -carotene rich foods or intrinsic labelled β -carotene foods in children with certain genotypes will reveal the magnitude of the effect of any newly discovered SNPs as well as the known SNPs. Also the role of high serum β -carotene concentration in retinol metabolism should be investigated as the β -carotene might be converted within and utilized by tissues directly, which can be identified by measuring retinol in tissue samples after dosing with labelled β -carotene.

Usefulness and success of the linear predictor developed in chapter 4 should be proven by incorporating it in new research, preferably in a different setting with higher inflammation. For comparison purposes, serum retinol by HPLC should be included in such studies, at least for a subsample of individuals. Retinol binding protein, transthyretin and C-reactive protein could be combined into one ELISA-assay for ease of measuring, but samples should be measured in duplicate to reduce variability. The provision of international reference material for these indicators would provide the possibility to compare between laboratories to increase transparency.

Introduction of biofortified yellow cassava should be combined with food based dietary guidelines, as our study showed that there is need for additional foods to fill the nutrient gap. These guidelines should be tested in the community for

feasibility and usefulness. We also showed that consumer's acceptance of yellow cassava was good; however this does not guarantee the same for a cassava eating population. Therefore acceptability should be assessed before yellow cassava will be introduced as well as extensive farmer trials regarding specific agronomic traits as yield and pest resistance should be conducted.

Public Health Importance

Considering the magnitude of the vitamin A deficiency problem in the world, yellow cassava can contribute to a higher vitamin A intake in populations in need, where cassava is consumed as a staple crop. While we found an overall modest effect with the yellow cassava varieties used, the newly bred varieties that contain up to $23\,\mu\text{g/g}$ fresh weight will most likely have a larger impact on vitamin A status. The varieties that were available for our studies can already be introduced in cassava consuming communities, since we have shown that these will have an impact on vitamin A intake and status. This will not only facilitate awareness and adoption of this yellow crop both by farmers and consumers but also increase familiarization to the new color and taste of yellow cassava and support the acceptability of the new varieties with higher β -carotene content in the near future. Because multiple micronutrient deficiencies often occur together, introduction of this new 'single-nutrient' crop should be accompanied by approaches that encourage changes to the traditional diets as well as introduce improved products such as other (bio) fortified foods or improved nutrient supplements to fill existing nutrient gaps.



The three combined proxy markers for vitamin A deficiency provide an easier method than HPLC to assess vitamin A status in resource-poor settings with very high accuracy. The decision rule provided enables researchers to better assess population vitamin A status, both at a lower cost and with more accuracy. This might allow inclusion of vitamin A status assessment in national surveys of countries. This method could replace measuring retinol by HPLC in circumstances that would not permit the use of difficult or expensive measurements, during population surveys with for example large sample sizes.

Conclusion

Overall we conclude that biofortified yellow cassava is efficacious in improving serum retinol concentrations in children in Kenya. New varieties with higher total β -carotene concentrations can be expected to have an even larger impact on vitamin A status. Yellow cassava was acceptable to this population, but introduction of yellow cassava has to be combined with additional food based dietary guidelines to improve the overall dietary nutrient adequacy of this community.

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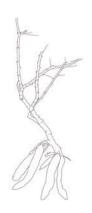
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Annex Statistical Analysis Plan Efficacy trial

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Statistical Analysis Plan

Overview

Summary

Vitamin A deficiency is still common in developing countries and has been proven difficult to combat. A promising approach is to replace common crops with varieties that are naturally richer in vitamin A, which is referred to as biofortification. For cassava, yellow β -carotene rich varieties have recently been introduced in Kenya, and these varieties are now ready to be tested for their efficacy to improve vitamin A status in humans.

Objective

The primary objective was to measure the effect of daily consumption of provitamin A biofortified cassava (providing 50% of the age-specific RDA) on vitamin A status in children aged 5-13 years with mild to moderate vitamin A deficiency in Kenya. The secondary objective was to 1) measure the effect of the intervention on immune function indicators 2) determine to what degree the serum retinol response to the intervention depends on serum concentrations of retinol and zinc at baseline; 3) determine the effect of the intervention on iron status and the functional indicator of gut integrity, 4) determine the mediating effect of SNPs in the BCMO1 gene on treatment outcome.

Study

Study population: This randomized controlled feeding trial was conducted in primary school children from three primary schools age 5-13 years living in the Kibwezi area in Eastern Kenya.

Intervention

After screening for eligibility and a 2-week run-in period (n=360) Children were randomly allocated to three different treatments:

- 1. 375 g of white cassava and a placebo capsule
- 2. 375 g of white cassava and a capsule containing 200 RAE of all-trans β-carotene
- 3. 375 g of yellow cassava targeting $\sim 50\%$ of the RDA for vitamin A and a placebo capsule.

Main study parameters/endpoints

The main outcome measure is serum retinol concentration at endline by means of ANCOVA. Other outcome measures include other vitamin A status indicators, iron status indicators, immune function indicators and gut integrity.

Preamble and scope

This plan is restricted to the statistical analysis of the primary study objective as formulated in the original proposal, i.e. to measure the effect of consumption of biofortified cassava on vitamin A status in children with low vitamin A status. It has been finalized before the treatment code was revealed. No interim analyses were planned or done.

Endpoints

The primary endpoint will be serum retinol concentration measured at the end of intervention. As secondary endpoints, we will consider the following indicators, all measured at the end of intervention:

Variable	Relative importance and role in interpretation of trial results
Serum all-trans-β-carotene concentration	Indicator of bioavailability of all-trans- β -carotene supplied by biofortified cassava
Vitamin A deficiency defined as serum retinol concentrations <0.70 µmol/L	Indicator of vitamin A status
Molar ratio of lactulose: mannitol in urine, and urine concentrations of lactulose and mannitol, following ingestion of lactulose and mannitol	Functional indicator of gut integrity (there are indications that vitamin A may reduce diarrhoea rates by improving gut integrity)
Serum concentration of retinol-binding protein and transthyretin	Indicators of vitamin A status
Haemoglobin concentration	Vitamin A may increase haemoglobin concentrations, although trial results have been conflicting, possibly because they are influenced by population-specific factors that determine the haematological response to vitamin A
Serum concentrations of neopterin, IL-2, IL4, IL10, TNF- α	Indicators of immune function that can be affected by vitamin A status



Definitions

Anemia is defined by hemoglobin concentration below 115 g/L for children from 5 to 11 years of age and by hemoglobin concentration below 120 g/L for children from 12-13 years of $age^{[1]}$.

Compliance to treatment is defined by the total amount of cassava that was eaten during the study as a fraction of the target amount eaten (age-specific) for the total duration of the study.

Compliance to attendance is defined as the total number of days attended the study as a fraction of the total duration of the study.

Inflammation is defined by either serum C-reactive protein concentration exceeding 5 mg/L or a1-glycoprotein protein concentration exceeding 1 g/L^[2]

Intestinal permeability or lactulose-mannitol-ratio (LMR) is measured by the ratio of percentage excretion of the ingested dose of lactulose (L) and mannitol (M) in the urine LMR. The normal ranges for the percentage excretion of lactulose, percentage excretion of mannitol and LMR are 0.10 – 0.52%, 3.92 – 29.0% and below 0.036, respectively (Algemeen Medisch Laboratorium, Antwerp, Belgium).

Iron deficiency is defined by serum ferritin concentrations below 15 μ g/L^[1] and serum soluble transferrin receptor concentration exceeding 21 nmol/L (1.55 mg/L)^[3]

Vitamin A deficiency is defined by serum retinol concentration below 0.7 µmol/L^[4].

Zinc deficiency is defined by serum zinc concentration below 9.9 µmol/L^[5].

Analysis plan

Sample size

Sample size calculation and power calculation was done before the start of the study and will not be reported on after the study because this is irrelevant for the interpretation of the results^[6].

Flow of the study participant and compliance per treatment group We will use a flow diagram to describe the flow of participants, as per CONSORT guidelines. Reasons for drop out after randomization will be given.

Description of exposure to intervention, by treatment group

- \circ Cassava raw and cooked: During study: trans- β -carotene, cis-carotene, total carotene, dry matter
- Placebo and active supplement: trans-β-carotene, cis-carotene, total carotene
- Daily cassava intake: During study: weighed food record
- Dietary intake assessment: 24h-recall: mid-study; VA, total energy and fat intake
- Compliance to the treatment and attendance

Data management

All data were double-entered, cross-checked for entry mistakes and stored in a central database in MS Access. Data analysis will be conducted using IBM SPSS (version 21). Distributions of dependent variables will be checked for normality and if needed transformed. Data will be checked for outliers and influence on group outcomes and be taken out if there is a good reason to mark it as an outlier. If not, the influence of the outlier on the results and interpretation will be assessed and reported. Missing values will be imputed if more than 10% of data are missing for primary outcome only and at endpoint only.

Description of baseline characteristics, by treatment group

We will produce a table to describe baseline characteristics for each intervention group. Data will be summarized as means (SD), median (IQR), or prevalence (%) as appropriate. The table will include the following variables:

Baselir	ne characteristics
Vital	and personal characteristics
	Age, years
	Sex
	Height-for-age z-score, SD
	Weight-for-height z-score, SD
	School
Infla	mmation markers
	Serum C-reactive protein concentration, mg/L
	Serum a1-glycoprotein protein concentration, g/L
	Inflammation
	Definition 1: Serum C-reactive protein concentration >5 mg/L
	Definition 2: Serum $a1$ -glycoprotein protein concentration > 1 g/L
	Definition 3: Serum C-reactive protein concentration > 5 mg/L or serum a 1-glycoprotein protein concentration > 1 g/L
Nutr	itional markers
	Vitamin A status, all children
	Serum retinol concentration, µmol/L
	Serum retinol-binding protein concentration, µmol/L
	Serum transthyretin concentration, µmol/L
	Vitamin A deficiency (serum retinol concentrations <0.70 μmol/L)
	Vitamin A status, restricted to children without inflammation 1
	Serum retinol concentration, µmol/L
	Serum retinol-binding protein concentration, µmol/L
	Vitamin A deficiency (serum retinol concentration <0.70 µmol/L)
	Zinc status μmol/L
	Serum zinc concentration, µmol/L
	Zinc deficiency (serum zinc concentration $< 9.9 \mu\text{mol/L}$)
	Iron markers, all children
	Haemoglobin concentration, g/L
	Anaemia (haemoglobin concentration < 115/120 g/L)
	Serum ferritin concentration, μg/L
	Serum soluble transferrin receptor concentration, mg/L
	Iron deficiency (serum ferritin concentration <15 μ g/L) (Soluble Transferrin receptor >21 nmol/L)
	Iron deficiency anaemia (iron deficiency and concurrent anaemia)
	Iron markers, restricted to children without inflammation ¹
	Serum ferritin concentration, μg/L
	Iron deficiency (serum ferritin concentration <15 μg/L)
	Iron deficiency anaemia (iron deficiency and concurrent anaemia)
Gene	etic markers
	SNPs related to vitamin A absorption and metabolism
	rs12934922, rs7501331, rs6420424, rs11645428, rs6513787, rs6564851,

 $^{^{1}}$ Defined as either serum C-reactive protein concentration > 5 mg/L or serum a1-glycoprotein protein concentration > 1 g/L



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Primary analysis

We will report effects as point estimates with corresponding 95% CIs; if this is not feasible, we will report p-values. In no circumstance will interpret results as 'significant' or report results accordingly (p<0.05).

We will visually inspect serum retinol concentration within intervention groups to assess whether it follows a t-distribution; if appropriate, we will transform this variable by log-transformation. We will use ANOVA and linear regression analysis to calculate group means, and to calculate the intervention effect as the difference in serum retinol concentrations between the groups (or, in case of transformation, as the ratio in these concentrations). In these analyses, we will include covariates for the stratified design and for possible effects of inflammation at the end of intervention to account for inflammation-induced changes in serum retinol concentration independently of vitamin A status. We will ignore the blocked design, because participants entered the trial in a single point in time, so that we do not expect the primary outcome to vary by blocks and the expected intra-block correlation is zero^[7]. To adjust for inflammation, the model will include terms for serum concentrations of both C-reactive protein and *a*1-acid-glycoprotein (both entered as continuous variables that may be log-transformed if giving a better model fit). This analysis will yield a crude intervention effect.

The primary analysis is per protocol and will be restricted to children with compliance to attendance and treatment of more than 80%; and without having experienced critical illnesses (major systemic disease) during the intervention period.

In the primary analysis, we will adjust for possible confounding. We anticipate the following baseline factors to be prognostic for the primary outcome (in decreasing order of expectancy): vitamin A status, SNPs related to β-carotene conversion to retinol, zinc status, iron status and sex. Confounding will be initially assessed by visual inspection ('eyeballing') of group differences at baseline in the factors (hence without testing for group differences[8]). In a subsequently step, we will use a full linear regression model as described in the preceding paragraphs, but also including the following baseline covariates: serum concentrations of retinol and zinc; hemoglobin concentration (continuous variables, centered around mean values); SNPs and sex (using dummy variables, with wild-type and males as reference categories, respectively). In addition, this full model will also include other factors for which there are substantial group differences at baseline but which we do not anticipate to be prognostic for the primary outcome. If confounding occurs (arbitrarily defined as a difference between crude and adjusted effect estimates exceeding 15%), we will use a backward elimination process to eliminate factors from the full model that do not substantially influence the effect estimate, and report the adjusted intervention effect obtained from the model with the terms remaining. In case confounding occurs, we will report the adjusted intervention effect as the one for primary attention, and the crude effect as supportive.

Secondary analyses

Full analysis set: To support the primary analysis, we will perform an analysis of the intervention effect on the primary outcome but on the set which is as complete as possible and as close as possible to the intention-to-treat ideal of including all randomized children ('full analysis set').

Subgroup analyses: We anticipate that the magnitude of the intervention effect on the primary outcome may be influenced by the following baseline factors:

Baseline factor	Justification
Vitamin A deficiency	Bioefficacy of provitamin A (i.e. the product of bioavailability and its conversion to retinol) is expected to be enhanced in vitamin A deficiency $[9]$
Zinc deficiency	Zinc is required for conversion of β -carotene to retinol ^[10] ; hence bioavailability of provitamin A may be reduced in zinc deficiency
SNPs related to vitamin A metabolism	Genetic variations in the BCMO1 gene may determine the bioconversion of provitamin A to vitamin A and thus the serum retinol response to biofortified cassava. There are few data on the prevalence of such polymorphisms in
	African populations, or their importance for provitamin A conversion ^[11]

To assess effect modification, we will initially assess intervention effects within strata defined by baseline status for either vitamin A or zinc (deficient versus replete), SNPs (genotype) We will subsequently assess possible effect modification directly using multiple linear regression models, either by evaluating product term effects and corresponding measures of statistical precision (95% CIs and p-values or by comparing models with and without multiple interaction terms by means of change in R2 test as an overall indicator of model fit). The results will be interpreted with caution and will be considered exploratory.

Secondary outcomes

Univariate analysis will be conducted by ANCOVA and MANOVA to determine any differences between treatment groups on the secondary outcome measures. Adjustment for covariates will be done as described for the primary outcome.

List	of	variables	assessed:

Variables	Baseline survey	During the intervention	End
Vitamin A and other carotenoid markers in serum			
Retinol concentration	X	0	Χ
Retinol-binding protein concentration	X	0	Χ
Cryptoxanthin concentration	X	0	Χ
Transthyretin concentration			
a-carotene concentration	X	0	Χ
Cis-β-carotene concentration	X	0	Χ
All-trans-β-carotene concentration	X	0	X
Serum iron markers			
Haemoglobin concentration	X	0	X
Ferritin concentration	X	0	X
Soluble transferrin receptor concentration	X	0	X
Vitamin E markers in serum			
a-tocoferol concentration	X	0	X
β-tocoferol concentration	X	0	X
γ-tocoferol concentration	X	0	Χ
Other nutritional markers			
Serum zinc concentration in serum	X	0	0
Urinary iodine concentration	X	0	0





Genetic markers			
SNPs related to vitamin A metabolism	0	0	X
SNPs related to iron metabolism	0	0	Х
Inflammation markers in serum			
C-reactive protein concentration	X	0	X
a1-acid glycoprotein concentration	X	0	X
Immunological markers in serum			
Neopterin concentration	Χ	0	X
IL-2 concentration	X ¹	0	X ¹
IL4 concentration	X1	0	X1
IL10 concentration	X1	0	X ¹
TNF-a concentration	X1	0	X1
Thyroid function markers in serum			
Thyroid-stimulating hormone (TSH) concentration	X	0	Х
Thyroglobulin (Tg) concentration	X	0	X
Tg-specific antibody concentration	0	0	X
Free thyroxine (fT4) concentration	Х	0	Х
Urine markers of gut integrity			
% excretion of Lactulose	0	0	X
% excretion of Mannitol	0	0	Х
% excretion of Lactulose/% excretion of Mannitol			X
Morbidity indicators			
Reported 14-day history of illness	Х	0	Х
Reported 14-day history of fever	X	0	X
Vital, socio-demographic and anthropometric data			
Age	Χ	0	0
Sex	X	0	0
Height	X	0	Х
Weight	X	0	X
School	Χ	0	0
Exposure markers			
Intervention group			
Concentrations of carotenoids ² in raw and cooked cassava	Rav	v once, cooked Da	aily
Dry matter content of raw and cooked cassava		v once, cooked Da	_
Daily cassava intake by weighed food record		v once, cooked Da	-
Dietary intake assessment by 24h-recall method		Mid-study only	
Vitamin A intake by 24h-recall method	Mid-study only		
Total fat intake by 24h-recall method		Mid-study only	
Total energy intake by 24h-recall method		Mid-study only	

X: sample/information collected; O: sample/information not collected

¹ Sub sample only n=100

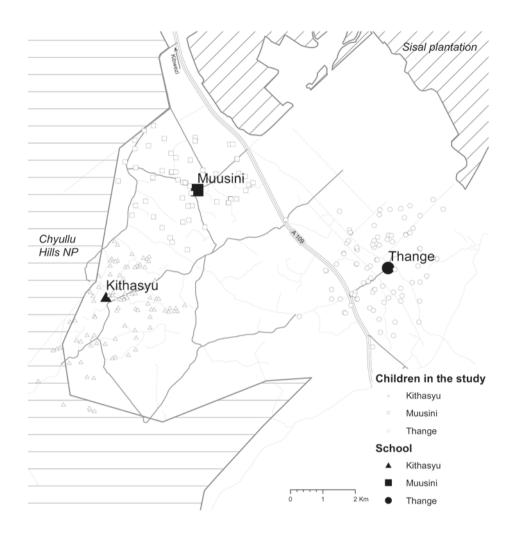
 $^{^{2}}$ Trans- β -carotene, cis-carotene, total carotene

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Map of the study area





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Summary



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Vitamin A deficiency is a major public health problem among vulnerable groups of young children and pregnant women in low-income countries. Biofortified provitamin A rich cassava has great potential to alleviate vitamin A deficiency and can be used as a complementary approach to other interventions. However, direct evidence whether biofortified cassava can significantly contribute to the vitamin A intake and status of populations is lacking. This thesis describes the research conducted to investigate whether this biofortified yellow cassava can improve the vitamin A status of children in Kenya.

The **first chapter** provides background information on vitamin A deficiency, biofortification and cassava, as well as the research questions in detail and a description of the study area.

Chapter 2 describes the randomized controlled efficacy study conducted with primary school children in Eastern Kenya. To study whether the daily consumption of biofortified yellow cassava can improve the serum retinol concentration of marginally deficient children, 342 children were randomized over 3 intervention groups:

- 1. White cassava and placebo supplement ('Control group')
- 2. Provitamin A-rich cassava and placebo supplement ('Yellow cassava group')
- 3. White cassava and β -carotene supplement (' β -carotene group')

The children consumed the boiled and mashed cassava 6 days per week for a duration of 18.5 weeks. The intervention increased serum retinol concentration in the yellow cassava and β -carotene supplement group compared to the control group with 0.04 µmol/L (95%CI: 0.00–0.07 µmol/L). Also serum β -carotene concentration increased with 524% µmol/L (95%CI: 448%–608% µmol/L) in the yellow cassava group and 166% µmol/L (95%CI: 134%–202% µmol/L) in the β -carotene supplement group. No evidence of effect modification by initial vitamin A status, zinc status, or polymorphisms in the β -carotene monooxygenase gene was found.

Biofortified provitamin A rich cassava has a distinct yellow color compared to the original white cassava. Therefore, we conducted a sensory and cultural acceptability study to find out whether the consumption of this new variety of cassava is acceptable to future consumers, which is described in **chapter 3**. Sensory acceptability was measured by replicated discrimination tests and paired preference tests among 30 children (7–12 yr) and 30 caretakers (18–45 yr) in three primary schools. Cultural acceptability was assessed with a questionnaire based on the combined model of The Theory of Planned Behavior and The Health Belief Model in one primary school among 140 caretakers of children aged 6 to 12 years. Both caretakers and children perceived a significant difference in taste between white and yellow cassava and both preferred yellow cassava because of its soft texture, sweet taste and attractive color. Almost all caretakers had the intention to prepare yellow cassava for their children. Future programs can increase this intention by:

- 1. Focusing on increasing knowledge about yellow cassava and vitamin A deficiency
- 2. Taking away worries about the bitter taste and color of the yellow cassava
- 3. Empowering the caretaker to have control to prepare cassava
- 4. Use activities like information sessions about yellow cassava with recommendations from health workers

The preferred reference method to measure vitamin A deficiency is based on serum retinol concentration measured by high-performance liquid chromatography

(HPLC). Because this method is often not available in low resource settings and is also costly and time consuming we studied other methods to assess vitamin A deficiency. **Chapter 4** describes the research that assessed the diagnostic performance of vitamin A deficiency proxy markers (retinol binding protein (RBP), transthyretin, retinol measured by fluorometry and RBP: transthyretin molar ratio) in detecting vitamin A deficiency as compared to low serum retinol by HPLC. Transthyretin and RBP showed the best diagnostic performance individually and when combined with C-reactive protein it provided the highest area under the curve of 0.98. A simple decision rule $\{(-15.277 \times [RBP, \mu mol/L] - 7.013 \times [Transthyretin, \mu mol/L] + 0.367 \times [C-reactive protein, mg/L] + 24.714) > -0.237\}$ yielded an unbiased estimate of the prevalence of vitamin A deficiency and can be used to assess the population burden of vitamin A deficiency.

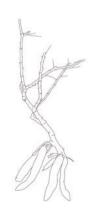


Chapter 5 describes a modelling exercise to formulate local food based recommendations and to evaluate whether providing yellow cassava as a school lunch contributes to nutrient adequacy. By using the linear programming tool OptiFood, food based recommendations including no school lunch, a school lunch with yellow cassava, or a school lunch with maize and beans, were compared on ability to fulfil nutrient adequacy. The final recommendations included a school lunch with yellow cassava that provided adequacy of most nutrients but not all. Even when combined with added fish and oil, the nutrient adequacy of fat, riboflavin, niacin, folate and vitamin A could not be ensured. The introduction of yellow cassava should be accompanied by approaches to improve the local diet with fish and oil, and alternative interventions should be formulated to fully eliminate nutrient inadequacy of schoolchildren in Kenya.

Finally, **chapter 6** discusses the main findings and conclusions of this thesis and puts these in a public health perspective including recommendations for possible future research. Overall we can conclude that yellow cassava is efficacious in improving serum retinol concentrations in sub deficient children with low vitamin A status. New varieties with higher total β -carotene concentrations can be expected to have an even larger impact on vitamin A status. Yellow cassava was acceptable to this population, but introduction of yellow cassava has to be combined with additional food based dietary guidelines to improve the overall dietary nutrient adequacy of this community.



Samenvatting



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een tekort aan vitamin A vormt een groot probleem voor kwetsbare groepen zoals jonge kinderen en zwangere vrouwen in gebieden waar armoede heerst. De introductie van gebiofortificeerde cassave die rijk aan pro-vitamine A is, zou in combinatie met andere maatregelen een goed middel kunnen zijn om tekorten aan vitamine A tegen te gaan. Er is echter nog geen hard bewijs dat deze gebiofortificeerde cassave een significante verbetering oplevert van de vitamine A inname en status bij deze groepen. In dit proefschrift wordt onderzoek beschreven naar het effect van gebiofortificeerde cassave op de vitamine A status van schoolkinderen in Kenya. Dit onderzoek is uitgevoerd bij kinderen van drie basisscholen in Kibwezi district in Oost-Kenya.

Het **eerste hoofdstuk** beschrijft de achtergrond van vitamine A tekorten, biofortificering en cassave, als ook de onderzoeksvragen. **Hoofdstuk 2** geeft een beschrijving van de gerandomiseerde en gecontroleerde studie die is uitgevoerd op basisscholen in Oost-Kenya. Deze studie omvatte 342 kinderen met een suboptimale vitamine A status op 3 basisscholen. De kinderen werden willekeurig verdeeld over 3 verschillende interventies:

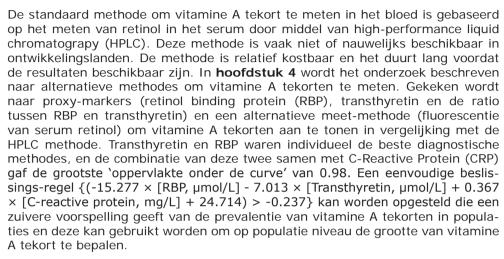
- 1. Witte cassave met een placebo supplement ('controle groep')
- 2. Gebiofortificeerde cassave met een placebo supplement ('qele cassave groep')
- 3. Witte cassave met β -caroteen supplement (' β -caroteen groep')

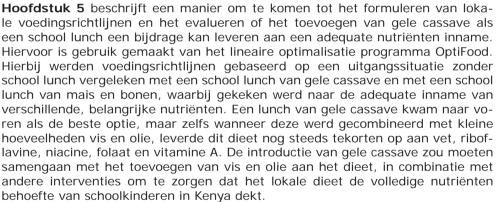
De kinderen kregen de cassave te eten in de vorm van 'puree' van gekookte cassave gedurende 6 dagen per week voor een periode van 18,5 week. Deze interventie leverde een verbetering op van het serum-retinol gehalte van de kinderen in de gele cassave en de β -caroteen groep ten opzichte van de controle groep van 0.04 µmol/L (95% CI: 0.00 – 0.07 µmol/L). Ook het serum- β -caroteen gehalte was verbeterd met 524% (95% CI: 448% - 608% µmol/L) in de gele cassave groep en 166% µmol/L (95% CI: 134%-202% µmol/L) in de β -caroteen groep. Er is geen bewijs gevonden dat de resultaten door de status in vitamine A en zink bij aanvang van de studie, of verschillende polymorfismes van het β -caroteen monoxygenase gen (BCMO1) beïnvloed zijn.

Gebiofortificeerde, pro-vitamine A rijke cassave is geel van kleur, hoewel de originele cassave wit is. **Hoofdstuk 3** beschrijft een studie naar sensorische en culturele acceptatie om uit te vinden of deze gele cassave acceptabel is voor de doelgroep. Sensorische acceptatie is bepaald door middel van zogenaamde 'herhaalde discriminatie testen' en 'gepaarde voorkeurs testen' bij 30 kinderen (7 tot 12 jaar) en 30 verzorgers (18-45 jaar) in drie basisscholen. Culturele acceptatie is bepaald door middel van vragenlijsten gebaseerd op een gecombineerd model (Theory of Planned Behavior & Health Belief model). Deze vragenlijsten werden afgenomen bij 140 verzorgers van kinderen in de leeftijd van 6 tot 12 jaar. Zowel verzorgers als kinderen vonden gele en witte cassave duidelijk verschillend en beide groepen hadden voorkeur voor de gele cassave, die lekkerder, zachter en aantrekkelijker van kleur gevonden werd. Bijna alle verzorgers gaven aan dat ze de gele cassave aan hun kinderen te eten zouden geven als deze beschikbaar kwam. Projecten die de introductie van gele cassave in de toekomst gaan verzorgen kunnen hun succes vergroten door:

- 1. Het verhogen van de algemene kennis met betrekking tot vitamine A tekorten en de rol van gele cassave hierin
- 2. Het wegnemen van zorgen met betrekking tot de bittere smaak en de kleur van gele cassave

- 3. De positie van de verzorgers te versterken, zodat deze meer invloed hebben op de keuze van het voedsel in het huishouden
- 4. Bijeenkomsten te houden over gele cassave en de aanbevelingen van gezondheids-werkers hierin te betrekken





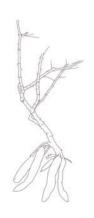
In **hoofdstuk 6** worden de belangrijkste bevindingen en conclusies van dit proefschrift beschreven en vertaald naar de mogelijke impact op de volksgezondheid. Ook worden aanbevelingen gedaan voor verder onderzoek. We kunnen op basis van dit onderzoek concluderen dat consumptie van gele cassave de vitamine A status van schoolkinderen met marginale status verhoogd. Nieuwe variëteiten met hogere β -caroteen gehaltes zijn in ontwikkeling en verwacht kan worden dat deze een nog sterker effect zullen hebben op de vitamine A status. Gele cassave werd geaccepteerd door deze populatie, maar de introductie van gele cassave zal samen moeten gaan met extra aanbevelingen om ervoor te zorgen dat het lokale dieet voldoende van alle nutriënten bevat.



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Acknowledgements



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And there I was, almost finished; finally, I only had to write the 'thank you part' of my thesis... I never imagined that it could be so difficult on one hand and so emotional on the other hand. Hard, because I don't want to forget anyone, and I had so much help... and emotional, cause it also reminds me of my PhD life that is almost over, almost 6 years full of hard work and interesting adventures....I also know that this part of the thesis is probably the part that is read the most and you might not even have a clue what I have been doing over the past 6 years. Well, if you want to remember anything, then it should be the yellow cassava, Kenya, and that the children called me Madame Muhogo (Kiswahili for cassava) and that they made fun of me as I did not even like the taste of cassava. A PhD thesis is never a one man show, but is the result of a combined effort of many. I will try to thank all of you, but please forgive me in advance if your name does not appear here, and know that I really appreciated what you did for me.

This work could not have been made possible without the help of all the school children in Makindu and Kibwezi disctrict in Kenya. I would like to thank the parents and teachers for allowing me to interrupt the daily school life so often. A special thanks to the children, teachers and parents of Muusini, Kithasyu and Thange Primary School, who participated in the cassava feeding trial. I hope the yellow cassava still grows in your gardens and please keep on sharing it so Kibwezi will be the new yellow cassava district of Kenya. I had great teams who organized the cooking and feeding very well; in Thange: Evelyne, Mwende, Jonathan, George, Pius, with headmaster mr Muli and deputy Clementine; in Muusini: Elizabeth, Priscah, Margaret, Hellen, Samuel, with headmaster Peter Munyao and deputy Eve Musyoka; in Kithasyu: Sammy, Mercy, Elizabeth, Makau with headmaster Daudi Munyao and deputy Faith Mwanza.

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My project was part of a larger program, called the INSTAPA project. Within INSTAPA there were many opportunities to collaborate with other research groups and organisations. We had fun annual meetings and therefore I would like to thank my fellow PhD INSTAPA students Marica, Colin, and Martin and also Busie, Erick, Eva, Chris, Lucy, Rita, Christophe, Pauline, Christele, Claire, Jane, Evariste, Jane, Richard, Waliou, Fatoumata, Polycarpe, Ines, Anne, Laurencia, Joseph and Wambui. Within INSTAPA I was working under the Kenya team at the Applied Nutrition Program of Nairobi University and therefore I would like to thank Alice for all her support while I was in the field and Joan, Ruto, Jane and Sophie for their help with many practical things.

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Rianna thank you for all the dedication with the samples and analyses and a special thanks to Brahim Tabali for coming all the way to Kibwezi to do the ELISA-assay in the hospital in Kibwezi. Karin, we had a lot of fun in the field, thanks for all your help and I hope we will write many more papers together! Geerke, our backs are not made for thesis writing, but I am sure you will do it quicker than I did, good luck! Vera, we occupied the agrotechnion very well together, it was fun to share the writing-path with you! Wanjiku, I am happy you can use parts of my data and that we shared some field work, I learned a lot from you and I am looking forward to our next papers! Ana Carla, Mexico was super, I am happy we did that and that you are here now, next one is yours! Catherine and Razak, both of you are back home, but our time together is not forgotten and I miss you both.

Victoire de Wild and Gloria Mbera, my two paranymphs, two wonderful women who stood by me during my PhD; Gloria who kept me going in the field in Kenya, and Victoire who kept me sane in the office in Wageningen. I am happy you are both here with me on the day of my defence and hopefully one day I can do the same for you.

Linda and Mathijs, thanks for allowing us to crash at your home that was so conveniently located around the corner of my office, and for taking care of my family when I was away or busy writing. Linda, you are the best second for J and R I could wish for. Tim and Vera, thanks for the interest, cakes and arrival dinners when we got back to NL all the time. It's great to share the love for adventures with you both. Lydia, Marleen, Akke, Anneke, Linda, Lilian, Fryske Famkes, Mapaseka and all my other friends I cannot name here, but who I have neglected, I hope you will see more of me in the future.

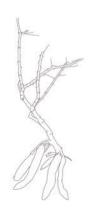
Haije en Ellie, thanks the for never ending support, helping out with R whenever you could, always visiting me in whichever country I was, for sitting under my chair, and with offering extra luggage allowances when travelling back to NL together. Frank en Hinko, Tinka en Jeannette, thanks for being there, and especially for not asking how the writing was going, and for proof reading and editing ideas of course. Super to work together! Frank en Ria, thanks for all support and the interesting talks we had about non-work-stuff, Maaike thanks for all your love, the calls to Kiwbezi and for forgiving me that I took your grandson and great granddaughter away again. Dimi and Pietje, thanks for being there, and in the next country you should visit us again!

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Ciao, Elise



About the author



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Elise Francina Talsma was born on 20 December 1978 in Zoeterwoude, the Netherlands. After completing secondary school at 'Bogerman College' in Sneek, she started the study Nutrition & Health (a bachelor and master program in one) at Wageningen University in 1998. As part of her study she conducted two master thesis research projects, the first one in the Hospital 'Isala klinieken' in Zwolle about the effect of a chromium rich diet on type 2 Diabetes Mellitus and the second one at the RIKILT Institute for Food safety in Wageningen on the identification of Ah-receptor agonist in citric products. As part of her master's program she did two internships; the first one at Unilever R&D Vlaardingen and Unilever Bangkok, Thailand, about the possible role of food products to reduce micronutrient deficiencies in Thailand; and the second one at Helen Keller International in Nepal on breastfeeding and complementary feeding practises in two rural communities.



After obtaining her master's degree in 2004, she worked as a research assistant for one year at the Division of Human Nutrition of Wageningen University on a randomized controlled trial about dairy intake and blood pressure. In 2005 she joined CARE International in Timor-Leste as a Junior Data Analyst and worked together with CARE and the Ministry of Health on a Supplementary Feeding Program for children under 5 years of age in three districts of Timor-Leste. In 2006 she started working for UNICEF Lesotho on Mother and Child HIV Care and Treatment related projects. In October 2008 she joined Wageningen University again to work on a PhD project about the efficacy of yellow cassava on vitamin A status in Kenya and she spent more than half of her PhD life living with her family in Kibwezi, Kenya, while conducting and managing the research as described in this thesis. She joined the educational programme of the Graduate School VLAG and was involved in teaching and supervising students at BSc and MSc level. She attended several national and international conferences and courses in the field of international nutrition. In 2011 she was a co-organiser (treasurer) of the PhD-tour for PhD-fellows affiliated with the Division of Human Nutrition to Mexico and the west coast of the USA. In 2013 she was selected and participated in the European Nutrition Leadership Programme in Luxembourg.

Since December 2013 Elise is working as a postdoctoral researcher in International Nutrition at the Division of Human Nutrition of Wageningen University where she first started with a project about the bioavailability of zinc from rice and milk and recently started to continue working with yellow cassava in a new HarvestPlus funded research project in Nigeria.

List of Publications

Publications in peer-reviewed journals

Talsma EF, Melse-Boonstra A, de Kok BPH, Mbera GNK, Mwangi AM, Brouwer ID (2013) Biofortified Cassava With Pro-Vitamin A Is Sensory And Culturally Acceptable For Consumption By Primary School Children In Kenya. PLoS One 8: 10.1371/journal.pone.0073433

de Waard WJ, Aarts JMMJG, Peijnenburg AACM, Baykus H, Talsma E, Punt A, de Kok TMCM, van Schooten FJ, Hoogenboom LAP (2008) Gene Expression Profiling In Caco-2 Human Colon Cells Exposed To Tcdd, Benzo[A]Pyrene, And Natural Ah Receptor Agonists From Cruciferous Vegetables And Citrus Fruits. Toxicol In Vitro 22: 396-410

Submitted publications

Talsma EF, Brouwer ID, Verhoef H, Mbera G, Mwangi AM, Demir AY, Maziya-Dixon B, Boy E, Zimmermann MB, Melse-Boonstra A. Biofortified Yellow Cassava And Vitamin A Status In Kenyan Children: A Randomized Controlled Trial. Submitted

Talsma EF, Verhoef H, Brouwer ID, Mburu-de Wagt AS, Hulshof PJM, Melse-Boonstra A. Proxy Markers Of Serum Retinol Concentration, Used Alone And In Combination, To Assess Population Vitamin A Status In Kenyan Children. Submitted

Talsma EF, Borgonjen-van den Berg KJ, Melse-Boonstra A, Kok FJ, Brouwer ID. The Contribution of Yellow Cassava To Nutrient Adequacy Of Primary School Children; The Use Of Linear Programming. Submitted

Published abstracts

Talsma EF, Brouwer ID, Mwangi AM, Zimmermann M,B Verhoef JCM, Melse-Boonstra A (2013) A Randomized Controlled Feeding Study With Provitamin A Rich Cassava Improves Total Carotenoid Intake In Primary School Children In Kenya. Ann Nutr Metab 63: 835-836

Talsma EF, de Kok BPH, Mbera GNK, Mwangi AM, Melse-Boonstra A, Brouwer ID (2013) Biofortified Cassava With Pro-Vitamin A Is Sensory And Culturally Acceptable For Consumption By Primary School Children In Kenya. Ann Nutr Metab 63: 836-836

Brouwer ID, Uwirigiyimana V, Talsma EF, Dossa RAM, Melse-Boonstra A (2013) Acceptability Of Yellow Cassava For Consumption By Schoolchildren In Didja, Benin. Ann Nutr Metab 63: 900-900

Brouwer ID, Ates B, Talsma EF, Dossa RAM, Melse-Boonstra A (2013) Identification Of Factors Determining Consumption Of Yellow Maize By School-Children In Kandi, Northern Benin. Ann Nutr Metab 63: 901-901

Melse-Boonstra A, Gichohi W, Talsma EF, Harryvan J, Boekschoten M, Towers W, Zimmermann MB (2013) Single Nucleotide Polymorphisms In The Tmprss6 Gene And Iron Status Indicators In Kenyan School Children. Am J Hematol 88: E87-E87

Talsma EF, Mwangi AM, Mburu-de Wagt A, Brouwer ID, Melse A, Verhoef H, Zimmermann MB (2009) Yellow Cassava (Yc) And Vitamin A Status In Kenyan Schoolchildren: Proposed Investigation Design. Ann Nutr Metab 55: 378-378

Overview of completed training activities

Disipline specific conferences and meetings	Country	Year
Experimental Biology	San Diego, USA	2014
Micronutrient Forum	Addis Ababa, Ethiopia	2014
Hidden Hunger Conference	Stuttgart, Germany	2013
International Conference on Nutrition ICN/IUNS	Granada, Spain	2013
Dies Natalis Wageningen University	Wageningen, the Netherlands	2013
The Malnutrition Puzzle: Embedding Nutrition in a Post-2015 Research Framework. Dr Judith Zwartz Lecture	Wageningen, The Netherlands	2013
African Nutrition Epidemiology Conference (ANEC)	Bloemfontein, South Africa	2012
Dutch Association of Africa Research; NVAS Afrika studiedag	Wageningen, The Netherlands	2012
INSTAPA Annual & Technical Meetings	Kenya, Benin, South Africa	2008-2012
International Conference on Nutrition ICN/IUNS	Bangkok, Thailand	2009
The Complexity of Impact Monitoring on MDG-1	Wageningen, The Netherlands	2009
Symposium; Maternal and Child Undernutrition: Challenges and Opportunities: Lancet Series	Utrecht, The Netherlands	2008
Disipline specific courses	Institute	Year
Good Clinical Practice in Research	Hospital Gelderse Vallei - Ede	2011
Epigenesis and Epigenetics-perinatal Nutritional Programming and Physiological Consequences	VLAG / WIAS	2011
Thysiological consequences		
NIHES Summer Course:	Erasmus University-NIHES	2009
3 0 1	Erasmus University-NIHES	2009
NIHES Summer Course:	Erasmus University-NIHES	2009
NIHES Summer Course: - Principles of Research in Medicine and Epidemiology	Erasmus University-NIHES	2009
NIHES Summer Course: - Principles of Research in Medicine and Epidemiology - Introduction to Data-analysis	Erasmus University-NIHES	2009
NIHES Summer Course: - Principles of Research in Medicine and Epidemiology - Introduction to Data-analysis - Methods of Clinical Research	Erasmus University-NIHES	2009
NIHES Summer Course: - Principles of Research in Medicine and Epidemiology - Introduction to Data-analysis - Methods of Clinical Research - Clinical Trials	Erasmus University-NIHES	2009

Production and Ese of Food Composition Data in Nutrition	VLAG	2009
General courses	Institute	Year
European Nutrition Leadership Program (ENLP)	ENLP	2013
Masterclass Analysis using R	VLAG	2012
Scientific Presentation: 'How to Present'	Mennen Training & Consultancy	2011
Philosophy and Ethics of Food Science and Technology	VLAG	2011
Advance statistics: Design of Experiments	WIAS	2010
Masterclass 'How to Write a World Class Paper'	WUR library	2010
Techniques for Writing and Presenting Scientific Papers	WGS	2010
Teaching and Supervising MSc Thesis Students	VLAG	2009
VLAG PhD Week	VLAG	2009
Information Literacy, including Introduction Endnote	WGS	2008
Optional courses and activities	Institute	Year
PhD Excursion 2011 Mexico & USA	VLAG	2011
Organising PhD Excursion 2011 Mexico & USA	VLAG	2010-2011
Development PhD Proposal	VLAG	2008-2009



Colophon

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