Université de Montpellier

HABILITATION A DIRIGER DES RECHERCHES

Ecole Doctorale GAIA

Malnutrition et carences des micronutriments chez des enfants

Frank Tammo Wieringa

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Devant le Jury composé de
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Prof Christine Benn
Prof David Thurnham
Prof Jacques Berger

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Chapter 1. Introduction of the candidate

1.1. Curriculum Vitea of the Candidate

Frank Tammo Wieringa was born on the 5th of September 1969 in Naarden, The Netherlands. He is Dutch, married to Marjoleine Amma Dijkhuizen, and together they have 3 children : Freija, Roelant and Pepijn.

Frank Wieringa joined IRD on the 1st December 2007. His current position at IRD is Chargé de Recherche (CR), Grade 1, Echelon : 8

He is affiliated to the so-called Unité Mixte de Recherche (UMR) 204: Nutripass (Nutrition et Alimentation des populations aux Suds) of IRD, which is based at the IRD-Centre in Montpellier, and directed by Jean-Pierre Guyot.

1.2 Research activities outline

I have been interested in research since the start of my university studies, in 1987. First, the research I was involved in was more biochemical in character, for example, research into the cytotoxicity of magainins (small peptides) from the skin of the African clawed frog, Xenopus laevis. However, already during my first year (1990-1991) internship at the Tropical Metabolism Research Unit (TMRU) in Jamaica, my interest moved towards nutritional sciences, and this has not changed in the 25 years that followed. TMRU was really the starting point of my nutrition research work, and I am still very thankful to our supervisors at that time: Michael and Barbara Golden.

The research done at TMRU (1990 – 1991) in Jamaica focused on severely malnourished hospitalized children, investigation in the course of a year various aspects such as the effects of aminoglycoside antibiotics on renal function, the development of a rat model for the effects of gentamicin on renal tissue, and various investigations into immune function compromise in severe malnutrion. The research was conducted under supervision of Dr Michael Golden and Dr Barbara Golden from the University of the West-Indies, and the University of Aberdeen, and Prof Ryk Luijken, from the University of Amsterdam.

The research work in Indonesia (1996 – 2001) focused on micronutrients, especially iron, zinc and vitamin A, and investigated micronutrient deficiency, miconutrient interactions, and effects of micronutrients on growth, metabolism, infection and immune function in infants and pregnant and lactating women. The research work was done under supervision of Dr. Clive West, Wageningen University and Prof Jos van der Meer, Radboud University Nijmegen, The Netherlands.

The research undertaken as post-doctoral fellow in Indonesia on tuberculosis and HIV (2002 – 2007) was under supervision of Prof. Jos van der Meer, Radboud University Nijmegen, The Netherlands.

During this time, I was also an invited lecturer at Mahidol University, as well as teaching at SEAMEO-TROPMED, University of Indonesia, Jakarta in the M.Sc. curriculum on vitamins and mineral deficiencies and the immune system.

In December 2007, I joined IRD as CR-1, and started working together with Jacques Berger in the Micronutrient Group of the UMR-204, Nutripass. I have continued working in this context until today

Overview of places where research was conducted before joining IRD (2007)			
Year(s)	University	Topic	Publications
1990-1991	University of Amsterdam, The Netherlands / University of the West-Indies, Kingston, Jamaica	Gentamicin pharmacokinetics in children with SAM ; lack of radio-graphic evidence in children with SAM	[1]
1991 - 1992	University of Amsterdam / Netherlands Cancer Institute, Netherlands	Cytotoxicity of magainins.	
1992	University of Amsterdam, The Netherlands	Prion diseases : A new class of infectious agents?	[2]
1996 - 2001	University of Wageningen / Radboud University Nijmegen, Netherlands / NRDC, Bogor, Indonesia	Vitamin A, iron and zinc deficiency in Indonesia (PhD thesis)	[3-13]
2003	UNICEF, Bangkok, Thailand	South-East Asia Trial of Iron and Zinc Supplementation in Infants (SEAMTIZI)	[14-16]
2004 - 2007	Radboud University Nijmegen, The Netherlands / Hasan Sadikin Hospital, Padjajaran University, Bandung, Indonesia	Nutritional supplements for patients with tuberculosis	[17, 18]

As also described below in Chapter 2, the work at TMRU, University of the West-Indies, Jamaica, focused on children with severe acute malnutrition (SAM) and the relationship between (mal)nutrition and infection. The research at TMRU focused on how children with SAM have different pharmacological handling of antibiotics, and how their reduced immune response leads to a lack of radiographic evidence of infection, especially pneumonia. Much later in my research career (2010), I returned to research on acute malnutrition and focused on the development and testing of locally produced, Ready-to-Use-Therapeutic Foods (RUTFs) for the treatment and prevention of SAM in Vietnam and Cambodia. In-between these periods, my research shifted towards micronutrient deficiencies, micronutrient interactions, and their impact on functional outcomes such as linear growth, immunocompetence and cognitive development. This line of research also continues until today. But the focus has slowly shifted away from technically studies using direct supplementation with vitamins and minerals towards more implementation-oriented studies on food fortification and food-to-food fortification.

1.3. Overview of research projects

It is hard to give a simple, straightforward overview of the research projects I have been involved in, as projects were developed based on available funding (unfortunately more and more an important constraint), available research collaborations, availability of students, and last but not least : new ideas or new policies that needed to testing. However, I think that the table below gives a global overview on how my research work has broadened and expanded over the last 2 decades.

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1.4. Supervision of students

Over the last 2 decades, I have supervised many MSc and PhD students. The table below will give an overview of these students, as well as their topics.

Name	Year*	University	Topic
PhD students			
Tran Thuy Nga	2009	Mahidol University, Thailand	Fortified biscuits for Vietnamese school children : effect on micronutrient status and parasite infection.
Thi My Thuc Luu	2013	National Institute of Nutrition, Vietnam	Vitamin A, B-vitamines and zinc in the recovery from diarrhea in Vietnamese children.
Jutta Skau	2014	Copenhagen University, Denmark	Preventing undernutrition in Cambodia: Assessing the effect of improved local complementary food on growth.
Brechje de Gier	2015	Vrije University Amsterdam, The Netherlands	Helminth infections and micronutrients in children.
Marion Fiorentino	2015	Université de Montpellier, France	Malnutrition in school-aged children and adolescents in Senegal and Cambodia: public health issues and interventions.
Pety Tor	2016	Université de sciences de Santé, Phnom Penh, Cambodia	Statut en acide folique dans les hemoglobinopathies chez les enfants des ecoles primaires à Kampong Speu
Khov Kuong	Autumn 2016	University of Copenhagen, Denmark	Fortified rice : Micronutrient stability, and impact on micronutrient status
Mulia Nurhasan	Autumn 2016	University of Copenhagen, Denmark	Fortified complementary foods in Cambodia: Willingness to pay, and impact on fatty acid status of infants.
Nguyen Song Tu	2017	National Institute of Nutrition, Hanoi, Vietnam	High-dose vitamin A post-partum: effect of timing and inflammation
Tran Khanh Van	2017	University of Copenhagen, Denmark	Fortified rice in Vietnam : introduction through village rice millers.
James Wirth	2018	University of Montpellier, France	Stunting, dietary intake and enteropathy
Daream Sok	2018	University of Copenhagen, Denmark	Impact of 6 months consumption of a fish-based LNS on micronutrient status
Bindi Borg	2018	University of Sydney, Australia	Impact of 6 month consumption of a fish-based LNS on anthropometry
Sanne Sigh	2018	University of Copenhagen, Denmark	Comparison of a locally produced RUTF with a commercial RUTF in treatment outcome in children with SAM
MSc students			
Marinka v.d. Hoeven	2005	Vrije University Amsterdam, Netherlands	Improving dietary intake in patients with HIV/AIDS in Bandung, Indonesia
Sabine van Elsland	2005	Vrije University Amsterdam, Netherlands	Improving dietary intake in patients with tuberculosis in Bandung, Indonesia
Suzanne vd Werff	2007	Vrije University Amsterdam, Netherlands	Acceptability of commercial high energy food products for PLWHA in Thailand
Marie Nguyen	2011	Université de Montpellier, France	Development and acceptability of a RUTF in Vietnam
Vinciane Parise	2011	Université de Montpellier, France	Vitamin A and inflammation
Jan Makurat	2012	University of Copenhagen, Denmark	Breastfeeding and iron status in Cambodian infants – WinFood study
Sou Chheng Ly	2013	Vrije University Amsterdam, Netherlands	Anthropometry and intestinal parasites in Cambodian school children - FORISCA
Remco Peters	2014	University of Copenhagen, Denmark	Development of a RUTF based on fish in Cambodia
Sanne Sigh	2016	University of Copenhagen, Denmark	Acceptability of a locally produced RUTF for the treatment of SAM in Cambodia
Ludovic Gauthier	2016	Université de Montpellier, France	Secondary analysis of the 2014 CDHS

* Year of defense of thesis, or expected year of defense of thesis.

Besides the direct supervision of the above-named students, have I also been involved in teaching in various MSc and PhD courses at SEAMEO-TROPMED, University of Indonesia, Jakarta, Indonesia (1998 – 2003), the University of Amsterdam, The Netherlands (2001 – 2004), University of Copenhagen, Denmark (2003 – 2006), Institute of Nutrition, Mahidol University, Bangkok, Thailand (2004 – 2007), the National Institute of Nutrition, Ministry of Health, Hanoi, Vietnam (2008 – 2012) and the National Institute of Public Health, Phnom Penh, Cambodia (2013-2014).

1.5. Collaborations

During the last decade, I have been very fortunate to be involved in a wide range of collaborations, both with institutes in Europe as well as in South-East Asia, which gave me the opportunity to learn and broaden my scientific scope. Collaboration with some of these institutes has led to long-standing collaboration, which included training of MSc and PhD students, collaborative project development, fund raising and execution of the projects. Below I have given a list of collaborations, with the projects involved, but this list is certainly not complete, but focuses on the most significant, productive and long-term collaborations.

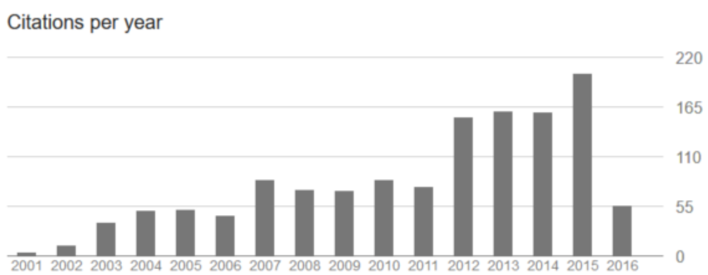
Institute	Projects	Students involved
Copenhagen University, Denmark	WinFood SMILING Vitamin A post-partum FORISCA LNS-Cambodia	Jutta Skau (PhD), Mulia Nurhasan (PhD), Khov Kuong (PhD), Daream Sok (PhD), Jan Makurat (MSc), Sanne Sigh (PhD), Remco Peters (MSc)
National Institute of Nutrition, Hanoi, Vietnam	SEAMTIZI RUTF-RUSF Vitamin A post-partum Fortified biscuits Fortified rice	Tran Thuy Nga (PhD), Marie Nguyen (MSc), Vinciane Parise (MSc), Nguyen Song Tu (PhD), Tran Khanh Van (PhD)
Mahidol University, Bangkok, Thailand	SEAMTIZI Fortified biscuit Vietnam SMILING	Tran Thuy Nga (PhD), Suzanne vd Werff (MSc)
Department of Fisheries Post-harvest Technologies and Quality control (DFPTQ), FiA, Cambodia	Winfood SMILING LNS-Cambodia FORISCA Longitudinal study	Jutta Skau (PhD), Mulia Nurhasan (PhD), Marlene Perignon (post-doc) Marion Fiorentino (PhD), Khov Kuong (PhD), Daream Sok (PhD), Jan Makurat (MSc), Sanne Sigh (PhD), Brechje de Gier (PhD), Remco Peters (MSc), Sou Ccheng Ly (MSc), Bindi Borg (PhD)
Vrije University Amsterdam, Netherlands	FORISCA SMILING	Brechje de Gier (PhD), Marinka v.d. Hoeven (MSc), Sabine van Elsland (MSc), Suzanne vd Werff (MSc)

1.6. Publications

All these projects have led to many scientific publications in peer-reviewed journals over the last decade. The table below gives an overview of most of my publications by journal and the number of citations for the publications combined.

Journal	Impact Factor (2014/2015)	Number of papers	Number of citations
Lancet	45.2	2	30
BMJ	17.4	1	0 (editorial)
Am J Clin Nutr	6.8	8	502
Nutrition Reviews	6.1	1	12
Antimicrob. Agents Chemother	4.5	1	1
Annals NY Acad Science	4.4	2	8
J of Nutrition	3.9	6	414
Nutrients	3.3	7	31
Br J Nutr	3.5	3	81
Plos One	3.2	8	87
Am J Trop Med Hyg	2.7	1	14
Eur J Clin Nutr	2.7	3	118
Nutr Journal	2.6	1	9
FNB	1.1	3	10
Afr J Reprod Health	0.3	1	7
total		48	1,324

Two other indicators to measure 'impact' are the h-index (h number of publication with h or more citations) and the i10-index (number of publications with ≥10 citations).



For me, the H-index is currently 16 and i10-index is 24. The number of citations/per year has risen over the last decade, as shown in the figure below (the year 2016 is only until April 1, 2016)

Five recent publications are attached to this dossier at the end.

2. Overview of the research conducted

2.1 Introduction.

Malnutrition is still one of the major global health problems, affecting billions of people in low-and-middle income countries, but also in high income countries. The term 'malnutrition' encompasses a wide range of disorders, ranging from severe acute malnutrition (SAM) to micronutrient deficiencies to obesity, which in itself is also a form of 'malnutrition'. In this thesis, I will be using 'malnutrition' especially to reflect 'undernutrition', and not so much 'overnutrition' as for the last 25 years my research has focused on undernutrition, especially on severe and moderate acute malnutrition, and deficiencies of micronutrients, and not so much on overweight and obesity.

3. Studies in severe and moderate acute malnutrition.

3.1. Introduction.

Acute malnutrition, which can be categorized as moderate (MAM; weight-for-height Z-scores (WHZ) between -2 and -3) or severe (SAM: WHZ<-3), is foremost caused by an imbalance between needs and requirements for energy and nutrients. Currently, there are 36 million children under 5 years of age with MAM in the world, and nearly 20 million children with SAM [19]. Children with SAM need to be treated with specialized therapeutic diets in combination with correct diagnosis and management of complications [20].

Severe Acute Malnutrition (SAM), which was formerly also known as Protein- Energy Malnutrition (PEM), is a complex syndrome, encompassing a wide range of disorders. The clinical presentation depends not only on nutritional deficiencies but also on additional stresses, like infections or toxins. The classical picture of a severely malnourished child is the so-called marasmic state. This form of SAM is essentially different from the kwashiorkor syndrome in that the former is clearly a result of inadequate energy intake, while the pathogenesis of the latter is more complicated, involving an overproduction of free radicals, resulting in pronounced metabolic changes and characteristic clinical features such as edema and a fatty liver[21]. In the marasmic-kwashiorkor syndrome, features of both extremes are found. The metabolic changes in SAM affect many biological functions including the clearance of antibiotics (described in 3.2.) and the immune system (described in 3.3.)

The complete treatment of SAM was usually hospital-based, with a specially formulated diet based fortified milk, F-75 during the stabilization phase until there were no more medical complications, followed by a F-100 based diet during the rehabilitation phase to promote rapid weight gain [20]. However, during these phases, which can take several weeks, the mother is required to stay with her child in hospital, which is a significant social-economic burden for the family as a burden for her toshe is not available for achieve her work and family-care activities. Moreover, hospital care predisposes to no-socomial infections[22]. Therefore, home-treatment is nowadays the preferred option for the treatment of SAM when complications have been addressed. But for this to be feasible and successful, an adapted version of the diet using special foods, so-called Ready-to-Use Therapeutic Foods (RUTF) is essential (described in 3.4. and 3.5.)

3.2. Gentamicin pharmacokinetics in malnourished children

SAM is commonly complicated by severe infections even though clinical signs of infection are often absent because of a general depression of the immune system. So, apart from dietary measures to restore nutritional status (also called nutritional rehabilitation), antibiotics are an important part of the treatment, especially in hospitalized cases. But considering the profound and divers metabolic changes that occur in SAM, it is not surprising that many physiological relations have been altered. Hence, in the malnourished state, drug pharmacokinetics are likely to be altered too, although data is scare. However, we showed clearly that gentamicin pharmacokinetics are significantly altered in SAM. Gentamicin is an aminoglycoside antibiotic which in the 1980’s and 1990’s was often used in SAM for its broad-range effectiveness and low cost. However it has ototoxic and nephrotoxic potential, and a narrow therapeutic range. In Jamaica, we obtained data on the pharmacokinetics of gentamicin in 15 children, aged 4 to 36 months with SAM. Of these children, 5 were diagnosed as marasmic, 5 as kwashiorkor and 5 as marasmic-kwashiorkor (according to the Wellcome Trust Classification). From the data we obtained, we could show that in children with oedema, the so-called volume of distribution for gentamicin was greatly increased, leading to sub-optimal peak and trough concentrations, and hence, most likely an ineffective treatment. In children without oedema who started recovery (nutritional rehabilitation phase), the clearance of gentamicin was increased, leading to adequate peak concentrations, but too low trough concentrations, again most likely leading to an ineffective treatment. These results highlight the difficulties in clinically managing children with SAM.

3.3. Lack of radiological evidence of pneumonia in children with SAM with fatal outcome.

One intriguing characteristic of SAM is that children often become ‘ill’, that is, develop fever or other symptoms of infection, during the recovery phase, while at admission, signs of infection are often absent. One possibility is of course that children become infected in the hospital. But a more likely explanation is that because of the severity of the malnutrition, the immune system is not capable of mounting an adequate response [23]. And without an adequate immune response, a child will not be able to produce a fever, which requires the production of cytokines such as interleukin 1 or 6) [24], nor be capable of producing a local immune response. And it is exactly this local immune response which is visible on e.g. chest X-rays that are used to determine whether a child has pneumonia or not. To test this hypothesis, we obtained chest X-rays from children with SAM, who had died within a week of taking the chest X-ray, and who on post-mortem examination had a positive bacterial culture from lung tissue, indicating pneumonia. We obtained also chest X-rays from children admitted to the chirurgical ward for non-infectious causes, and asked a radiologist to read all chest X-rays for signs of pneumonia. We were able to retrieve 14 X-rays from children who died of SAM with a culture done from lung tissue, 6 of them positive. From those 6, 3 had normal chest X-rays and 3 showed consolidation, which could be regarded as consistent with pneumonia. In the 8 children with negative cultures, all chest X-rays were normal. Hence, this small study showed that ~50% of the children with SAM were incapable of mounting an inflammatory response[1].

3.4. Development of Ready-to-Use-Therapeutic Foods (RUTF) in Vietnam and Cambodia

3.4.1. Introduction

As described above, home treatment of children with SAM is becoming more and more the preferred program policy, as home-based treatment programs such as IMAM (Integrated Management of Acute Malnutrition) has been proven to be as successful, or even more, as hospital-based treatment. One the reasons behind this success has been the development of so-called 'Ready-to-Use Therapeutic Foods' (RUTF). RUTF is a generic term including different types of foods, such as spreads or compressed products suitable for feeding severely malnourished children. The formulation of RUTF was derived from F-100, which was traditionally used in the treatment of SAM. RUTF is a homogeneous mixture of lipid-rich and water-soluble foods, with a very high energy density (~23 kJ/g or 5.5 kCal/g), and that can be eaten without further preparation. For example, the most well-known RUTF spread called PlumpyNut is a lipid-rich paste, resulting in an energy-dense food that resists microbial contamination[25]. This RUTF is a mixture of milk powder, vegetable oil, sugar, peanut butter, and powdered vitamins and minerals. As the name implies, RUTFs do not need to be prepared in any way prior to consumption, making it practical for use where cooking fuel and facilities are limiting constraints, and guaranteeing at the same time the hygiene and correct composition of the food. Although PlumpyNut was successfully introduced in Africa, an acceptability trial with Plumpynut in Cambodia showed that the product was not well accepted by Asian children[26]. Therefore, together with UNICEF and the National Institute of Nutrition, Ministry of

Health, Vietnam, we started in 2009 with the development of a locally produced RUTF, adapted to the taste of Vietnamese children.

3.4.2. Acceptability of a locally produced RUTF in pre-school children in Viet Nam

In Viet Nam, about 7% of the children under 5 years of age have acute malnutrition, that is have a weight-for-height of <-2 Z scores. For Viet Nam this account for an annual caseload of wasted children ~780,000, including >275,000 children with SAM[27].

Viet Nam started to implement the integrated management of acute malnutrition (IMAM), which includes community-based treatment of acute malnutrition from 2010 onwards. For this, the availability of a RUTF acceptable for local taste and preferences was essential. The composition of the RUTF had to fulfill the strict UNICEF requirements, while at the same time we need to take the nutritional qualities of the ingredients, local availability and price into account. After an initial exploratory phase, the research team decided to develop a RUTF in form of a compressed bar instead of a paste-like product like PlumpyNut, because paste-like foods were uncommon in Vietnam. Also, the compressed bar resembled a popular Vietnamese snack (Banh Dau Xanh or green bean cake), improving the chances for producing an acceptable product. The final product, termed HEBI (High Energy Bar for IMAM), contained mung and soy beans, rice, sesame, sugar, whole milk powder, whey protein, vegetable fat, vegetable oil and a vitamin-mineral premix.

To assess the acceptability and organoleptic qualities of the product, we conducted a trial in 2010 among school children to compare the acceptability of HEBI to Plumpy'nut®[28]. The trial was a randomized, cross-over design study in which children received both products subsequently for 2 weeks, two times per school day. As Plumpy'nut® is a paste and HEBI a bar, the study could not be blinded. The RUTF was given at 9:30 am and at 3 pm, replacing the normal snack meal which is provided at those times.

At baseline, the children (n=69) could be classified as underweight but not acutely malnourished, with a mean WAZ of -2.12 and a mean WHZ of -1.81. Acceptability of both products was good and both RUTF scored high for organoleptic qualities (i.e. for color, smell, taste, palatability), with more than 75% children scoring 'good' for both products. Overall intake of Plumpy'nut® was higher, even though children showed a higher reluctance towards Plumpy'nut® than to HEBI

(P<0.05), with overall reluctance to both products being high especially during the first week. All anthropometrical indices increased significantly over the 4 week intervention period which was reflected in significant higher WAZ, HAZ and WHZ scores at the end of the study, with increases of 0.34, 0.05 and 0.48 Z-scores respectively (P<0.01 for all). MUAC increased by 0.5 (± 0.3) cm (P<0.001) over the intervention period. There were no difference between the 2 RUTF with respect to impact on anthropometric indices.

Because of these results, the composition of HEBI was slightly adjusted to improve palatability, and the effectiveness of HEBI in the home treatment of SAM tested in a subsequent intervention study (see 3.5.).

3.4.3. Acceptability and impact of a locally produced RUTF in HIV positive children and adults in Vietnam

Malnutrition is also a common feature of HIV and AIDS, and low body mass index (BMI) at the time of diagnosis of HIV infection presents a significant risk factor for mortality. Chronic weight loss occurs in around 20 percent of patients with AIDS, and severe weight loss is one of the strongest indicators associated with morbidity and reduced survival, even with antiretroviral therapy (ART). Evidence shows that interventions with both macro- and micronutrients are likely to delay the progression of disease, improve the outcome of opportunistic or concomitant infections, improve the effectiveness of ART, reduce side effects of medication, and improve quality of life [29, 30]. But this evidence mainly comes from studies conducted in Africa, and data are lacking from Asia. Viet Nam's HIV prevalence rate is low, at 0.43 percent among people 15–49 years old in 2009, although this figure still means a significant number of HIV-positive individuals in a population of over 85 million. Ready-to-use therapeutic food (RUTF) has been found to be a highly effective and relatively inexpensive intervention to treat severe acute malnutrition (SAM) among people with or without HIV. Because of the high cost of importing RUTF in Viet Nam, and with funding from the U.S. President's Emergency Plan for AIDS Relief (PEPFAR)/Viet Nam and technical support from the Food and Nutrition Technical Assistance Project (FANTA), IRD collaborated with NIN in 2011 to study the comparative acceptability of Plumpy'nut® and the locally produced HEBI among children and adults with HIV[31]. The study focused on assessing the acceptability of the product in terms of consumption a predefined about, as well as organoleptic properties such as taste and texture. Because acceptability, taste, and texture preferences may differ between children and adults, two studies were conducted simultaneously, one with 80 HIV-positive children (aged 3 to 7 years) attending the National Pediatric Hospital in Hanoi and one with 80 HIV-positive adults attending the Hospital for Tropical Diseases in Ho Chi Minh City. The study was a randomized crossover study in which HIV-positive participants were randomly divided into 2 groups of approximately 40 each. Each group was randomly assigned to receive one product, either Plumpy'nut® or HEBI, during the first 2 weeks and the other product for the subsequent 2 weeks. In addition, a third group of 40 HIV-positive participants in each study was randomly assigned to be in the comparison group, which did not receive any products during the study. Plumpy'nut® and HEBI servings were designed to provide the same amount of energy (1 serving Plumpy'nut (92 grams) = 500 kcal; 1 serving HEBI (1 bar) = 500 kcal). The child dose prescribed was one serving of RUTF (500 kcal/day), and the adult daily dose prescribed was two servings of RUTF (1,000 kcal/day). Subjects were provided with a 2-week supply of the RUTF to which they were randomly assigned at the beginning of the study and were given another 2-week supply of the other RUTF for the second 2-week period. Children consumed 69 percent of the 2-week dose of HEBI and 65 percent of the 2-week dose of Plumpy'nut® ($p=0.13$). Many children did not respond when prompted to choose between the two different RUTF for preference. They did not, on average, rate the organoleptic properties of the two RUTF any differently. Children who did indicate a preferred product tended to choose HEBI (65%) over Plumpy'nut® (35%) ($p=0.058$). Adults consumed 91 percent of the 2-week dose of HEBI and 81 percent of the 2-week dose of Plumpy'nut® ($p= 0.059$). The 10 percentage point difference between HEBI and Plumpy'nut® in the results observed among adults was not statistically significant at $p <0.05$ but could be meaningful in clinical settings. Adults HIV patients overwhelmingly preferred HEBI to Plumpy'nut® (79% vs 21%, $p <0.0001$). Significant gains in WAZ and BMI-for-age Z-score were observed over the 4-week study period among children who received RUTF in comparison with children who did not receive RUTF ($p=0.014$ and 0.036 respectively). In adults, significant weight and BMI gains were also observed over the 4-week study period among adults who received RUTF in comparison with the adults who were randomly assigned to the control group and who did not receive RUTF ($p=0.004$ and 0.0048, respectively). Overall, these findings confirm the findings of the acceptability trial in the pre-school children with unknown HIV status that the provision of a RUTF for a relative short period (4 weeks) can have a significant effect on anthropometry[31].

3.4.4. Development and acceptability of RUTF in Cambodia

Like in Vietnam, the acceptability of readily available, imported products to treat or prevent malnutrition was low in Cambodia, as shown by the low acceptability of PlumpyNut[32]. To test whether the products developed in Vietnam would have potential as a tool to treat or prevent malnutrition in Cambodia, we conducted an acceptability trial of the Vietnamese RUTF, as well as the conventional therapeutic products BP100 and CSB++, and a peanut-based RUTF called Eezee Paste (produced by Compact Norway). Two products from Vietnam were tested: a bar and a newly developed paste. Hence, in total 5 different products were compared in the acceptability trial in 2013. A total of 66 care takers and 67 children participated in the acceptability trial, with complete data available for 114 participants. Participants were asked to taste all the 5 products, and then pick the best and the worst products. As best product, 34.2%, 28.9% and 21.2% of the participants chose Eezeepaste, HEBI-paste and HEBI-bar respectively, with <5% of the participants choosing the currently used product for the prevention of malnutrition : CSB++, as best product. Only 10.5% choosing the currently used product for the treatment of malnutrition : BP-100 as best product. Reversely, 65% and 20.2% of the participants (>85% combined) chose CSB++ and BP-100 respectively as the worst product, showing that the products presently used by the Ministry of Health in Cambodia and international organizations for the prevention and treatment of malnutrition have a low acceptability among the Cambodian population.

Therefore, we started with the development of a locally produced RUTF in Cambodia, based on local taste preferences and ingredients available. We conducted a first acceptability trial with the new product in pre-school children in 2014 to test the locally produced paste again BP100. The new product initially contained 8 - 12% dried fish. The higher amounts of fish resulted however in a very strong taste, so for the acceptability trial, a paste with 8% of dried fish was prepared. As fish, Siamese Mud crap (trey riel) was used, a small fish which is caught in huge quantities in the months December and January in the Tonle Bassac river.

Table 8. Final composition pilot LC-RUTF

Ingredients	%
Siamese Mud Carp (Trey Riel)[1]	8.00
Mung bean[2]	14.50
Soy bean[2]	15.00
Rice[3]	5.70
Sugar	14.95*
Maltodextrin[4]	12.60
Oil (Canola)[5]	15.50
Vegetable Shortening[6]	12.25
Mineral-vitamin premix	1.5**
Total:	**100.00**

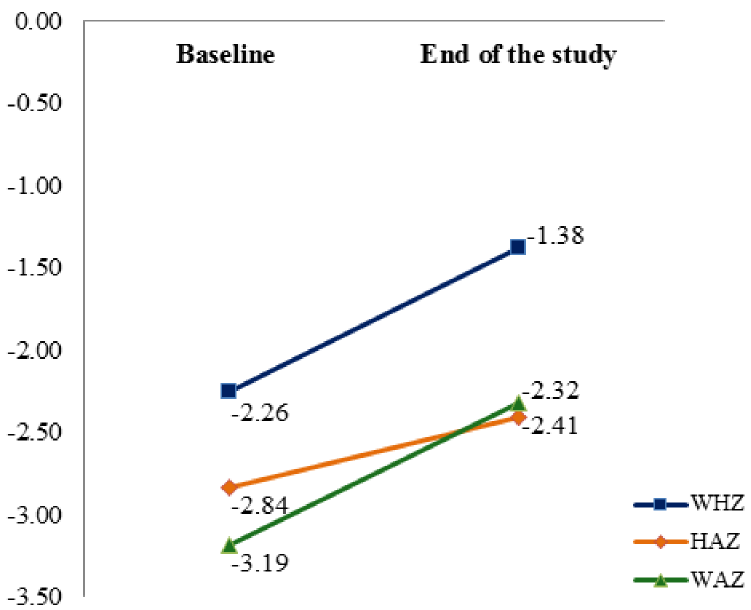
The first results of the acceptability trial were however not so positive, with the locally produced RUTF being not very well accepted by the children (neither was the commercial product, BP-100).

Therefore we changed the composition of the product again, and changed also the form of presentation (a paste in a wafer) as well as the production process of the paste. Although the final product still contains 8% dried fish, a new acceptability trial in 2015 showed a much higher acceptability, and the new product scored much higher than e.g. CSB++ when given to mothers with young children. Therefore, in March 2016, an intervention trial was started with the new product to test whether the product could prevent young children from becoming malnourished. At approximately the same time (December 2015), a trial was started in the National Pediatric Hospital to test whether the new product was as good as the current RUTF (BP-100) given to children for treatment of malnutrition. Results of both studies are expected by the end of 2016 or early 2017.

3.5. Impact of Ready-to-Use Therapeutic Foods on treatment outcome in Vietnam

After the acceptability of the locally produced RUTF had been proven in Vietnam, both in a general population of pre-school children, as in specific populations of HIV+ children and adults, the impact of the RUTF on treatment outcome had to be proven too. During the acceptability trial in the pre-school children, HEBI was a little less consumed than Plumpy'nut® because the bar was drier than the paste. Before scaling-up production of the Vietnamese RUTF, some readjustments were made to improve the Vietnamese to assess the effectiveness of the local RUTF on weight gain and recovery rate using the RUTF in home treatment of acute malnutrition. The effectiveness trial was preferred to an efficacy trial because IMAM guidelines were being introduced at the same time, and we urgently needed data on the impact of HEBI under real world conditions. The study was conducted in 2011 in Kon Tum province. This province was chosen since the national guidelines for IMAM were already implemented in Kon Tum city and hence health workers had already been trained on the diagnosis and treatment of SAM. The district next to Kon Tum city, Sa Thay, still followed the old guidelines (nutritional education only) and was chosen as 'control group', in order to show that the new policies were more effective in treating SAM than the old policies. All children between 6 and 59 months of age with acute malnutrition without complications were eligible for inclusion in the study. Children from Kon Tum city with acute malnutrition were randomly assigned to receive either HEBI or Plumpy'nut® for 8 weeks. Caretakers of children from Sa Thay district with acute malnutrition were given nutritional education by the nutrition/medical staff of the health center on how to prepare meals for wasted children using local foods available at the communes, and were invited to return the health center for a weekly check-up. If children did not show signs of nutritional improvement (weight gain) within 2 weeks, they would be switched to the new guidelines and be randomized to receive either HEBI or Plumpy'nut®. Indeed, none of the children included in the initial control group showed any weight gain during the first 2 weeks, and all of them were randomly allocated to the 2 RUTF for an 8 weeks period.

The 8 week intervention resulted in a significant improvement of all anthropometric indices with



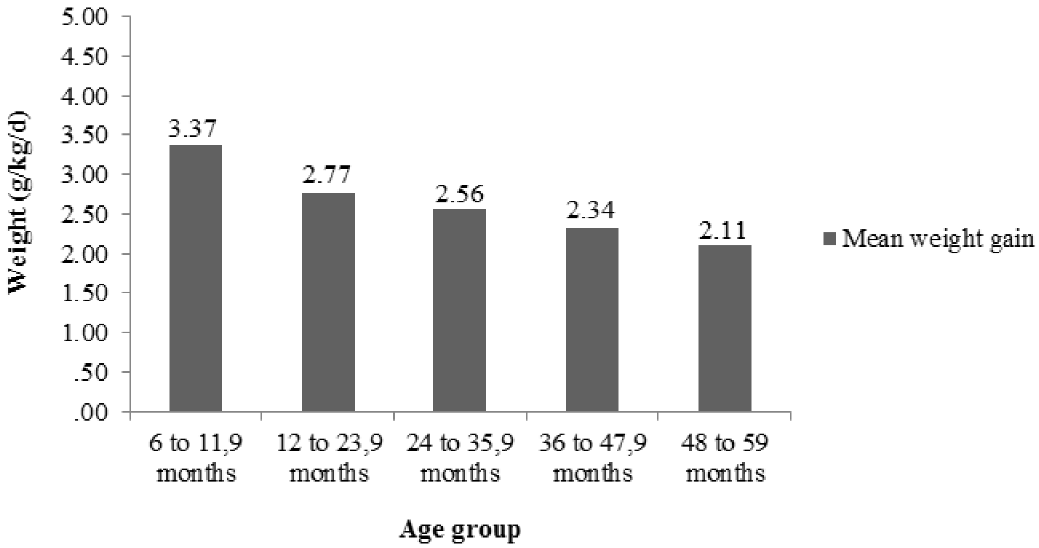
increases of +0.88, +0.43 and +0.88 Z-scores for WHZ, HAZ and WAZ respectively.

Changes were different based on the nutritional status of the child: children with SAM gained more weight than children with MAM and stunted children showed a higher gain in height than non-stunted children.

The local RUTF was as well appreciate as Plumpy'nut® concerning the color and smell, and was preferred in terms of taste and palatability, showing that the improvement of the formulation had been effective. Consumption of HEBI was higher than for Plumpy'nut®, however it was also easier to share than Plumpy'nut®, so the consumption calculated may not reflected the "real" consumption by the child admitted in the study. Our main outcome was of course weight gain. Although weight gain was significant, with a mean increase of ~3.0 g/kg/d for children with SAM, it was lower than the target of at least 4 g/kg/d at set by WHO.

	PN group	HB group	Control then PN	Control then HB
Weight gain (in kg)	1.17 ± 0.59	1.22 ± 0.49	1.22 ± 0.66	0.86 ± 0.47
Weight gain (g/kg/d)	3.11 ± 1.69	2.76 ± 1.23	2.41 ± 1.06	1.91 ± 1.02

One reason for this could be sharing of the RUTF within the family, which was reported mainly for HEBI. The presentation of HEBI as little cubes, and the resemblance to Banh Dau Xanh facilitate sharing. Also, weight gain per body weight was higher in younger children as compared to older children.



Significant height gains were also found for children older than 2 years old (HAZ +0.29 Z score). This HAZ increase most likely represents catch-up growth in those moderate stunted children. There has been some debate on whether catch-up growth is possible after 2 years of life, but this study shows clearly that increases in height are possible in children older than 2 years of age[33].

However, despite the lower than expected weight gain per body weight, recovery rate was >70%, with at the end of the study 66% of the children having neither SAM nor MAM. As a result of this study, the local RUTF was included in the IMAM policies and it was decided that the implementation should be rolled out over more provinces. By 2011, a functional food production unit for Ready to

Use Therapeutic Food was established through NINFOOD, Department for Food Technology from the National Institute of Nutrition. By end of 2013, IMAM activities were carried out in 11 provinces. The facility at NINFOOD currently has the production capacity to meet the need for HEBI production, with pressing of the cubes no longer done manual but being automated.

In September, 2012 a General Manufacturing Practices (GMP) inspection was conducted by an expert from UNICEF Supply Division in Copenhagen to ensure compliance with international and national quality assurance and food safety standards. Currently, NIN, IRD and UNICEF are further developing the product to also include supplementary foods (RUSF) to be used in the prevention of severe malnutrition by targeting children with moderate acute malnutrition (WHZ between -2 and -3 Z-scores). Indeed, in 2012, an acceptability trial for a new RUSF to manage MAM was conducted among preschool children in Bac Giang Province, which showed that the RUSF was highly acceptable, and the willingness to pay for the product was such that social-marketing can be considered. During the trial, valuable feedback was obtained regarding the flavour, smell, and consistency of the product, which was used to optimise the RUSF formulation. An efficacy trial of the RUSF, testing the RUSF alone or in combination with WASH, was started in Bac Giang Province in 2013, combining the provision of RUSF with behavioural change messages including WASH.

3.6. Impact of Ready-to-Use Therapeutic and Supplementary Foods on treatment and prevention of malnutrition in Cambodia.

Together with UNICEF, the National Nutrition Program (NNP) of the Ministry of Health, the Department of Post-harvest Technologies and Quality Control (DFPTQ) and the Universities of Copenhagen and Sydney, we are currently conducting 2 studies for the treatment and prevention of malnutrition in Cambodia. As described above, the paste is packed in a rolled wafer, and given to children to eat directly or to be mixed with the rice porridge prepared by the mother.

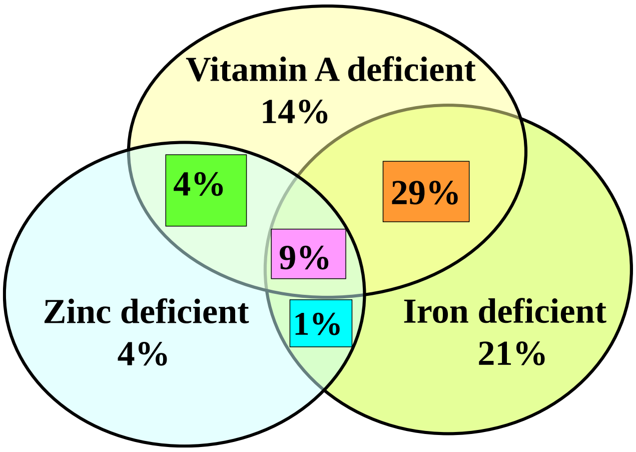
In total, 140 children with SAM will be recruited for the treatment trial, and 540 infants, aged 6 – 12 months, will be recruited for the prevention trial. Results of both studies will be available by the end of 2016 or early 2017

4. Micronutrient deficiency

4.1. Introduction

Malnutrition can be caused not only by insufficient quantity of food, leading to acute malnutrition as described above, but also by poor food quality, leading to a lack of specific nutrients. The quality of the diet relates to the nutrient content of the diet, including the balance of amino acids but also to e.g. fatty acid and micronutrient content. "Micronutrients" is a collective term used to describe vitamins and minerals, which are required in only small amounts by the body. Qualitative inadequacy of diets leading to deficiency of various micronutrients is highly prevalent, although signs or symptoms of these micronutrient deficiencies are often lacking. Micronutrient deficiency is therefore also referred to as the "hidden hunger"[34].

Most intervention and research efforts in the field of micronutrient nutrition have concentrated on deficiencies of vitamin A, iron and iodine, in concordance with the priorities set by the World Summit for Children in 1990, the Ending Hidden Hunger Conference in 1991, and the International Conference on Nutrition in 1992. However, other micronutrients such as zinc, copper, vitamin B1 (thiamin) and vitamin D, also warrant attention, not in the least because of the interactions among



many micronutrients, and their interwoven roles in metabolic processes. People are likely to be deficient with respect to more than one micronutrient concurrently, as the same causative factors can underlie the etiology of deficiency for different micronutrients, as we showed already in 2001 for Indonesian infants and their mothers [35]. As depicted in the graph, Indonesian infants were more likely to have >1 micronutrient deficiency (total 43% of the children) than only 1 micronutrient deficiency (total 39%).

A cereal-based diet, rich in phytate and low in animal products is common in most developing countries, including all countries in SE Asia. Such a diet predisposes to insufficient absorption of both iron and zinc[36]. Furthermore, the amount of retinol and the absorption of provitamin A carotenoids from such a diet will be low. This is illustrated by the finding that vitamin A deficient infants and lactating women in Indonesia are 2 to 4 times more likely to be deficient in iron and/or zinc than vitamin A sufficient infants and women[35]. However, besides being just part of the same, inadequate, diet, micronutrients also interact which each other. Both synergistic (e.g. iron and vitamin C) as antagonist effects have been reported[16, 37, 38]. For a more in-depth description of micronutrient interactions please see chapter 5.

Severe micronutrient deficiency often gives distinct signs and symptoms, and can be directly life threatening, but is not very prevalent anymore in most countries in SE Asia. The manifestations of marginal micronutrient deficiency often appear minor and not specific, but can impair development and increase the risk of morbidity and mortality. However, marginal deficiency of various micronutrients is much more prevalent than severe deficiency, affecting health, growth and development of populations in an insidious way. Therefore, the overall burden of marginal deficiency on health and development is much greater than that of severe deficiency. The most vulnerable groups in the population are preschool children, and pregnant and lactating women. Marginal to moderately severe iron deficiency for example affects over 30% of all women. Micronutrient requirements during pregnancy and lactation are increased, and diets in developing countries often do not meet these higher requirements [39]. Therefore during pregnancy and lactation micronutrient stores often become depleted, leading to impairment of micronutrient status, affecting both mother and infant. Marginal deficiency of various micronutrients in children has direct consequences for psychomotor development, immune function and growth [40, 41]. For example, children with marginal vitamin A status have a higher morbidity and mortality of infectious diseases. Many studies have shown that deficiency of various micronutrients during pregnancy is associated with unfavorable pregnancy outcomes such as maternal mortality, congenital abnormalities and low birth weight[42].

4.2. The prevalence of micronutrient deficiencies in vulnerable groups in SE Asia

One of the first step to address micronutrient deficiencies is to understand the magnitude of the problem. This can be done through simple research of literature, compiling what has been published, or by conducting elaborate field studies, sometimes involving thousands of subjects in order to obtain a reliable indication of national or provincial prevalence of deficiency in selected micronutrients. I have been involved in both types of research in the last years.

For the EU-funded SMILING project (Sustainable Micronutrient Interventions to controL deficiencies and Improved Nutritional status and General health in Asia), we conducted two state of the art systematic reviews regarding the micronutrient status of women of reproductive age and young children in 5 South-East Asian countries: Vietnam, Laos, Cambodia, Thailand and Indonesia. Results of these 2 reviews showed that deficiency of several micronutrients are still prevalent in South-East Asia. However, data for most countries are rather dated, hence there is an urgent need for up-to-date evidence on micronutrient status of vulnerable groups in SE Asia.

	Cambodia	Indonesia	PDR Laos	Thailand	Vietnam
Vitamin A status					
- serum <0.7 µmol/L	0.3%	-	-	1.7%	1.6%
- nightblindness	5.1%	<1%	12%	-	-
Iron					
- Anaemia	55%	23%	36%	25%	28%/12%
- Iron deficiency anaemia	8%	-	23%	-	-
Zinc (serum conc.)	-	-	-	-	67%
Iodine Median urinary conc.	-	190 µg/L	-	180 µg/L	83 µg/L (all)

Estimated prevalences of deficiency in children – SMILING project

For example, the data on vitamin A and iron status in Cambodia dates from 2000, whereas data on anemia was available up to 2014. But as we showed in the first Cambodian National Micronutrient Survey as well as in the national survey in Women of reproductive age in Cambodia, most of the anemia in Cambodia is not due to either iron or vitamin A deficiency, hence anemia is not a good indicator of iron status in the Cambodian context.

Although these reviews of literature are often informative on which micronutrients might pose a public health problem in terms of deficiency, policy makers often require biochemical data to base new policies on. We have been conducting 2 national micronutrient surveys in Vietnam and in Cambodia to assess the micronutrient status of women of reproductive age and children, and although these micronutrient surveys most often confirmed the literature searches or the dietary intake data, sometimes we were in for surprises.

The first study was conducted in Vietnam in 2010, in a collaboration with GAIN and the National Institute of Nutrition, Ministry of Health, Vietnam. The object of this survey was to establish nationally representative data on the prevalence of anemia, iron, vitamin A, zinc, folate, vitamin D and calcium deficiency among women of reproductive age (WRA) and preschool children in Vietnam[43-45]. For this micronutrient survey, we followed the national representative food consumption survey which had been conducted in Vietnam the year before (2009). For this 2009 survey, a stratified proportionate to population size (PPS), two-stage cluster sampling procedure had been used. For the 2010 Vietnam Micronutrient Survey, households were re-visited that had participated in the 2009 national food consumption survey. In total, 112 clusters (56 rural and 56 urban), comprising of 1680 households, were selected at randomly (out of the 512 clusters used in the 2009 food consumption survey). The survey showed, perhaps surprisingly, a relative low prevalence of anemia and iron deficiency, both in children and in women of reproductive age. In contrast, prevalence of zinc deficiency was very high.

Micronutrient status indicator	Prevalence of deficiency in Women of Reproductive age	Prevalence of deficiency in children <5 yrs of age
Hemoglobin (Anemia)	11.6	9.1
Ferritin (<15 μg/L)	13.7%	12.9
Iron Deficiency Anemia	5.4%	3.2
Plasma retinol (<0.70 μmol/L)	1.6%	10.1
Zinc deficiency (<9.9 μmol/l)	67.2%	51.9
Vitamin B12 deficiency (<148 pmol/l)	11.7%	-
Vitamin D deficiency (<30 nmol/l)	17.3%	20.3%

Overview of the prevalence of micronutrient deficiency in Vietnamese women of reproductive age and children. From Laillou et al [44, 45].

In 2014, we had the opportunity to revisit households that had participated in the 2014 Cambodian Demographic Health Survey to conduct the first National Micronutrient survey in Cambodia (CMNS2014). The Micronutrient Survey was linked to the Cambodian Demographic Health Survey (CDHS) 2014, but was not an integral part of it, that is, data for the CMNS2014 was collected after the data collection of the CDHS2014 has been done. The CMNS2014 was implemented in 1/6 of the clusters selected for the CDHS 2014, and collect data only in women and children <5 yrs of age. Blood, urine, and stool samples were collected from women who had given birth in the five years preceding the survey and from children age 6-59 months.

The survey identified 1,048 mothers and 1,358 children who were eligible for the micronutrient study. However, about one in four mothers and their children (27 percent and 24 percent, respectively) refused to participate in the micronutrient survey. Refusal rates were considerably higher in urban areas than in rural areas for both mothers and children. Moreover, the quantity and/or quality of the specimens were not always sufficient for the laboratory analyses, especially in the case of children. Hence, final data were available for ~740 mothers and ~790 children. Like in Vietnam, the prevalence of iron deficiency was much lower than expected on anemia prevalence. Whereas >40% of the mothers and >50% of the children was anemic, iron deficiency was only present in 2.6% of the mothers and 3.3% of the children.

	Urban	Rural	Total
Hemoglobin level			
Anemia[1]	46.23	43.50	43.89
Number of mothers	71	415	485
Ferritin <15 mg/L	3.61	2.40	2.55
sTfR >8.3 mg/L	31.57	34.23	33.89
Number of mothers	96	642	738

[1]All pregnant mothers with hemoglobin <11.0 gram per deciliter (g/dl) and all nonpregnant mothers with hemoglobin <12.0 g/dl, after adjustments for altitude and for smoking status, if known, using formulas in CDC (1998), are classified as anemic. The hemoglobin level was measured in two-thirds of the households in each cluster during the main DHS data collection. This is why the number of women in the table is lower for hemoglobin level.

	Urban	Rural	Total
Hemoglobin level			
Anemia[1]	42.2	55.1	53.4
Number of children	87	573	659
Ferritin <12 µg/L	5.0	3.1	3.3
sTfR >8.3 mg/L	44.0	47.9	47.5
Number of children	88	705	793

[1] All children with hemoglobin levels below 11.0 g/dl (after adjustment for altitude using formulas in CDC, 1998) are classified as anemic. Hemoglobin levels were tested in two-thirds of the households in each cluster during the main DHS data collection.

In contrast, the prevalence of hemoglobinopathies was very high, with >50% of the mothers having a hemoglobin pattern that could be described as abnormal (HbA<95%). Hemoglobinopathy E was the most common form found, with 28% of the mothers being heterozygote, and another 8% being homozygote HbE.

Table 17.3 Type of hemoglobin among mothers by residence

Among mothers age 15-49 who have at least one child born since January 2009, percent distribution by type of hemoglobin, according to residence, Cambodia 2014

Type of hemoglobin	Urban	Rural	Total
Normal hemoglobin[1]	46.6	39.6	40.5
Heterozygote hemoglobin E[2]	25.5	28.4	28.0
Homozygote hemoglobin E[3]	7.9	5.7	6.0
Other forms of hemoglobin[4]	19.3	23.2	22.7
Missing	0.8	3.1	2.8
Total	100.0	100.0	100.0
Number	96	643	739

[1] Hemoglobin A1 >95 percent
[2] Hemoglobin E between 20 percent and 30 percent
[3] Hemoglobin E >80 percent
[4] Any other forms of hemoglobin spectrum

In the children, iron and zinc deficiency, hookworm infection and hemoglobinopathy were significantly associated with anemia, whereas in the women only iron deficiency was associated with anemia. Iron deficiency anemia (IDA) was prevalent in children <2 years, but in older children and women, the prevalence of IDA was <5%. In the children, prevalence of iron, vitamin A, vitamin B12 or vitamin B9 deficiency was <10%.

Hence, only in children <2 years of age, iron deficiency anemia was of some concern, whereas in older children, the prevalence of iron deficiency anemia rapidly declined.

		Age (months)			
	6 – 11	12 – 23	24 – 36	36 – 48	>48
N	27	92	115	111	204
No anemia	44.4% [a,b]	37.0% [b]	54.8%[a]	60.4% [a]	60.8% [a]
IDA[1]	11.1% [a,b]	15.2% [b]	4.3% [a,c]	1.8% [c]	2.5% [c]
Anemia, non-ID	44.4% [a]	47.8% [a]	40.9% [a]	37.8% [a]	36.8% [a]

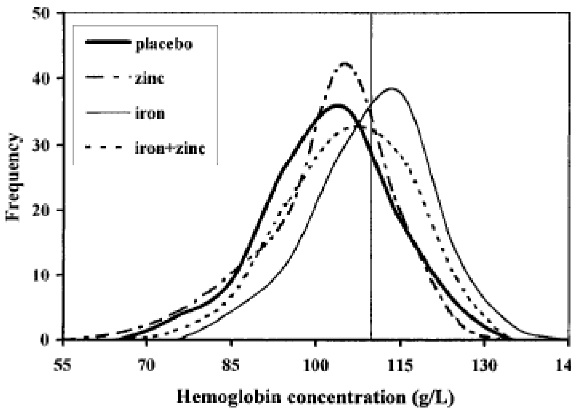
These results raise fundamental questions on what causes the high prevalence of anemia in Cambodian women, as even in women with a normal hemoglobin pattern, and with normal iron stores, anemia prevalence was >30%.

4.3. Strategies to improve micronutrient status of vulnerable groups: Effects of supplementation on micronutrient status.

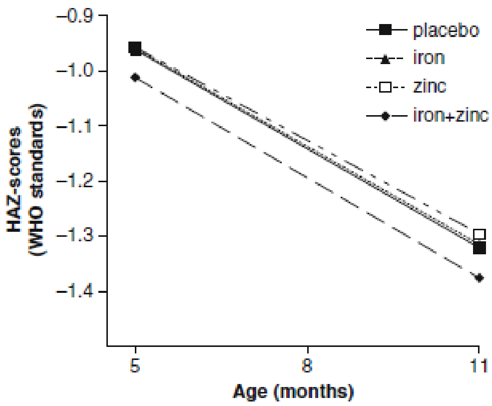
The most direct way to correct a micronutrient deficiency is by providing that specific micronutrient, either through medical intervention (injection / infuse) or as an oral supplement. Indeed, until around the 1990's, these strategies to improve micronutrient status where the most common utilized and imbedded in many national health policies. Examples include iron and folic acid supplements for pregnant women, or the high-dose vitamin A capsule (VAC) programs for young children. Especially the VAC programs were hailed as 'golden bullet' in the 1990's, as research showed that the programs could reduce child mortality by as much as ~25%. Perhaps surprisingly, the impact of VAC programs on vitamin A status itself was harder to prove, and often not present. Similar, although iron and folic acid supplements for pregnant women were standard practice throughout the developing world, it proved hard to find benefits for the newborn, with a 2009 Cochrane review reporting no significant effects on important infant health outcomes such as premature delivery, low birth weight, perinatal death, or infant hemoglobin concentrations at 6 months of age[46]. Of course, one can only expect an improvement in micronutrient status (or the biomarker for it), when there is deficiency. For instance, data from the 2014 Cambodian Micronutrient survey showed that 51% of the children under 5 years of age were anemic. Anemia is often used as proxy-indicator for iron deficiency. However, in the Cambodian Micronutrient Survey, only 6% of the children had iron-deficiency anemia. Hence improving iron status will do little to reduce the prevalence of anemia in this context.

But when iron deficiency is an issue, like in young children in Indonesia, supplementation is the most direct intervention to correct this. In one study in Indonesia, we supplemented infants from 4 months of age with a syrup containing iron, zinc, iron+zinc or placebo daily for 6 months to see whether zinc supplements could stop growth faltering. Indeed, all the children in the study experienced serious growth faltering (dropping from an average -0.9 Z score at 4 months of age to an average -1.3 Z-score at 10 months of age), meaning that they were not able to keep up with the international growth curves. However, it was not that the zinc or iron supplements were not effective. Iron and zinc status improved significant in the infants receiving iron and zinc, and the prevalence of anemia dropped from ~66% in placebo group to 28% in the iron only group (and to

46% in the iron and zinc group). In these infants, iron deficiency was a major issue with >50% of the infants having ferritin concentrations <15 μg/L, indicative of depleted iron stores. The effect of supplementation is perhaps best illustrated by examining the distribution curves of hemoglobin concentrations for the different intervention groups, which shows that the whole distribution curve in the iron-only group is shifted towards higher hemoglobin concentrations. A similar pattern was seen for plasma zinc concentrations.



Later, when results from 3 other studies were combined with this study as part of the SEAMTIZI trial, we showed that these results were not unique for Indonesia, but also were applicable for Vietnam and Thailand. In the pooled data from those 4 sites, both iron and zinc supplements were effective in improving iron and zinc status respectively, although the combination of iron and zinc was less effective. But growth faltering took place in all infants, regardless of whether they received iron or zinc supplements[47]. Hence, although micronutrient status was improved by the supplements, functional outcomes such as growth were not.



Impaired growth is one of the most consistent signs of malnutrition. Studies on nutritional growth impairment indicate that the onset of linear growth faltering is probably within a few months of birth, and that the most sensitive period for intervention is prior to 18 months of age [48]. But in infants and young children, not only energy and/or protein deficiency will lead to growth impairment, but also a poor dietary quality leading to deficiency of one or more micronutrients, with especially zinc deficiency being linked to growth impairment [49]. However, providing only 1 micronutrient might not be enough to stop growth faltering, as growth is the end-result of complex metabolic processes, involving many nutrients. Hence, it was perhaps not surprising that we did not find an effect of zinc supplementation in our studies in Indonesia on growth [50].

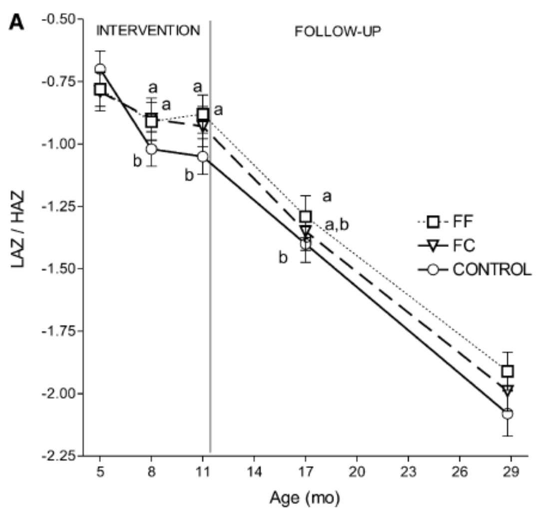
4.4. Food-based to improve micronutrient status in women and children

4.4.1. Introduction

Food based approaches to improve the micronutrient status of populations include strategies ranging from food fortification to food-to-food fortification to dietary diversification. Fortification of foods, and particular staple foods such as wheat or rice, is a cost-effective tool to improve the micronutrient status of populations at risk for micronutrient deficiencies. Indeed, of the tools currently available to alleviate nutritional deficiencies among vulnerable groups, food fortification is the most promising and cost-effective[51]. Over the last decades, an increasing number of food products are being fortified with vitamins and/or minerals worldwide. However, in South-East Asia fortified foods are not yet widely consumed. Even the consumption of iodized salt, which was a mandatory fortified food in e.g. Vietnam has decline dramatically over the last decade[52, 53]. Rice is the main staple food for most populations in South-East Asia, accounting for >60% of daily energy intakes. Hence, fortification of rice could be an efficient and cost-effective way to improve micronutrient status in women and children in South-East Asia.

4.4.2. Complementary foods with improved nutrient density.

A special case of food fortification is complementary foods. Infants from 6 months of age onwards and young children under the age of 2 years need special foods (complementary foods) with a high nutrient density, to complement the breastmilk. The traditional complementary foods given to young children in South-East Asia are mostly a rice-based watery porridge, with an energy and nutrient density that is too low to be able to sustain the high growth velocity during the first years of life, leading to growth faltering and in the end stunting. Therefore, providing more nutrients, both macro- and micronutrients, might have a better effect to stop growth faltering, than providing only some selected micronutrients, as was done in the SEAMTIZI trials. Indeed, this is what we showed both in Vietnam as in Cambodia. The provision of a complementary food with a higher energy and nutrient density than the traditional porridge could stop the growth faltering during the first months of life. In Vietnam, provision of complementary foods stopped the growth faltering over the 5 month intervention period, and this effect remained after the end of the intervention, even though growth faltering started immediately again after the intervention[54]. The intervention consisted of either a complete complementary food (FF) or a food complement, which was added to a traditional



porridge, thereby increasing micronutrient content and energy density of the porridge. The intervention also improved micronutrient status of the infants, hence in this study, there were improvements both in biochemical indicators of micronutrient status as in functional outcomes.

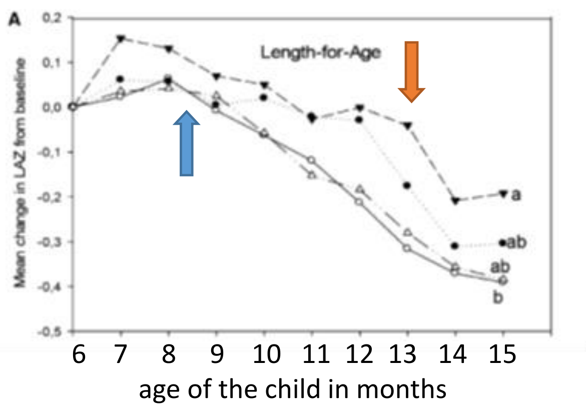
TABLE 2 Prevalence of micronutrient deficiencies in Vietnamese infants at baseline and at the end of the interventions including either a FF or a FC or following traditional feeding practices (C)[1]

	FF		FC		C		
	n	%	*n*	%	*n*	%	P^2
Anemia (Hb <110 g/L)							
Baseline	157	62.4 (54.8, 70.0)	135	71.9 (64.2, 79.5)	134	66.4 (58.4, 74.4)	NS[3]
Final	120	36.7 (28.0, 45.3)$^{a,\#}$	106	28.3 (19.7, 36.9)$^{a,\#}$	123	56.1(47.3, 64.9)^{b}	<0.001
Low ferritin (<12 μg/L)							
Baseline	157	30.6 (23.4, 37.8)	134	20.9 (13.9, 27.6)	133	27.8 (20.7, 36.0)	NS
Final	120	14.2 (7.9, 20.4)$^{a,\#}$	106	14.2 (7.5, 20.8)^{a}	122	58.2 (49.0, 66.5)$^{b,\#}$	<0.001
High TfR (>8.5 mg/L)							
Baseline	157	3.2 (0.4, 5.9)	134	2.2 (0.0, 4.7)	133	2.2 (0.0, 4.7)	NS
Final	120	0.8 (0, 2.5)	106	1.9 (0.0, 4.5)	123	4.9 (1.1, 8.7)	NS
Low retinol (<0.70 μmol/L)							
Baseline	157	79.6 (73.3, 85.9)	135	77.4 (69.9, 84.1)	131	83.2 (77.7, 89.6)	NS
Final	120	39.2 (30.4, 47.9)$^{\#}$	106	34.9 (25.8, 44.0)$^{\#}$	123	45.5 (36.7, 54.3)$^{\#}$	NS
Low zinc (<9.9 μmol/L)							
Baseline	157	66.2 (59.5, 72.9)	135	64.4 (56.9, 71.9)	134	67.2 (59.5, 74.9)	NS
Final	119	36.1 (27.1, 45.0)$^{a,\#}$	105	42.9 (33.3, 52.4)$^{a,b,\#}$	121	52.9 (44.3, 61.5)$^{b,\#}$	0.027
ID (PF <12 μg/L or TFR >8.5 mg/L)							
Baseline	157	32.5 (25.1, 39.8)	134	20.9 (14.0, 27.8)	133	28.6 (20.9, 36.3)	NS
Final	119	13.4 (7.3, 19.6)$^{a,\#}$	105	15.2 (8.4, 22.1)^{a}	120	57.5 (48.6, 66.4)$^{b,\#}$	<0.001
IDA							
Baseline	157	21.7 (15.2, 28.1)	134	17.9 (11.4, 24.4)	133	18.8 (12.1, 25.4)	NS
Final	119	6.7 (2.2, 11.2)$^{a,\#}$	105	3.8 (0.1, 7.4)$^{a,\#}$	120	37.5 (28.8, 46.2)$^{b,\#}$	<0.001

[1] Values are mean percentage (95% CI). Means in a row with superscripts without a common letter differ, $P < 0.05$; #different from baseline, $P < 0.05$.
[2] *P*-value represents Pearson's χ-square.
[3] NS, $P \geq 0.05$.

From Phu et al. J Nutition 2010 [55]

It is tempting to suggest that if the Vietnamese study had continued for a longer period, e.g. for 9 months or longer, the effect on linear growth might have been more sustained. However, in Cambodia, we couldn't stop growth faltering from occurring during a 9 month study. This study (WinFood) had a different set-up. Instead of providing a group of children with a traditional porridge to serve as a control group, all children received an improved porridge, with a higher energy and nutrient density than the traditional porridge. The objective of the study was to test whether we could make a complementary food completely made with local ingredients (the WinFood product: °) or from local ingredients plus an imported vitamin and mineral premix (the WinFood lite product: •). We compared these 2 products against the 'gold standard' in complementary foods : CSB++ (corn-soy blend ++, which contains milk powder: ▾) and a product



often used for older children CSB+ (without milk powder: Δ). As can be seen in the figures, in the 2 complementary foods with only macronutrients without additional animal protein (CSB+) or with micronutrients with perhaps a poor bioavailability (WinFood), growth faltering started within 3 months of the intervention (blue arrow), whereas in the 2 products with both animal protein and additional micronutrients from a premix (CSB++ and WinFood lite), growth was sustained for around 6 months, before growth faltering occurred (orange arrow). However, no product was capable to support optimal growth for the full 9 months of the intervention.

4.4.3. Fortified biscuits for school children in Vietnam

Whereas much attention is given to the nutritional status of children under the age of 5, school-aged children are often neglected. For example, a survey in 2007 in North Vietnam showed an intake of vitamin A in school children of between 39-50%, and for iron of between 42-54% of the Vietnamese recommended daily intake (RDI)[56]. In Vietnam, like in most South-East Asian countries, the diet consists mainly of rice, vegetables and legumes. Animal foods are an excellent source for several micronutrients such as iron, vitamin A, zinc and vitamin B12, as nutrient density and bioavailability from these animal sources is much higher compared to plant sources. But even though the intake of animal foods has been increasing gradually over the last 10 years in Vietnam, it still is only a small contribution to overall intake. Besides malnutrition, intestinal parasite infection is often prevalent too in schoolchildren. For example, in our study in school children in North Vietnam, >80% of the children had at least 1 intestinal parasite, mainly ascaris or trichuris. Intestinal parasite infestation prevalence was lower in school children in Cambodia (18%), but here >95% of the infestation was due to hookworm. We assume that the half-yearly deworming campaigns in Cambodia have contributed to the low prevalence of ascaris and trichuris infection, but have been unsuccessful against hookworm infections, as the albendazole or mebendazole is only given as a single dose.

We conducted two studies using similar biscuits fortified with multiple micronutrients to identify feasible, affordable solutions to improve micronutrient status and health of school children[57, 58]. Biscuits were chosen as food vehicle for the studies because of the ease of distribution, familiarity with the product and low price. And biscuits targeting school-age children were already being produced in Vietnam, so no new product needed to be developed. At the time of the studies, there was no school meal program in Vietnam, hence simply fortifying a product already being used in school canteens was not an option, as was done later in Cambodia with fortified rice. Both studies showed an impact on micronutrient status, with the fortified biscuits improving micronutrient status. One study not only looked at biomarkers for micronutrient status, but also looked at functional outcomes such as growth and cognitive scores[58], and the interaction between micronutrient fortification and intestinal parasites.

TABLE 3 Biochemical outcomes of the school children who completed the intervention of no treatment or baseline deworming with or without 4 mo of multi-micronutrient fortification and effect sizes[1]

Outcome	Placebo	MMF	Alb	MMF+Alb	Estimated effect sizes[5] (95%CI)		
					MMF	Alb	*P*-interaction
n	118	114	117	118			
Hb, *g/L*	120.1 ± 8.0	121.2 ± 7.3	119.9 ± 7.0	122.2 ± 6.2	1.87[b] (0.78, 2.96)	0.50 (−0.59, 1.59)	0.950
Plasma ferritin,[2] *μg/L*	59.9 (41.5, 85.1)	63.6 (46.1, 85.5)	60.5 (43.5, 84.7)	69.5 (52.9, 91.4)	7.5[b] (2.8, 12.6)	2.8 (−1.6, 7.6)	0.750
Plasma TfR, *mg/L*	5.4 ± 1.5	5.5 ± 1.4	5.5 ± 1.3	5.2 ± 1.2	−0.139 (−0.32, 0.04)	−0.089 (−0.26, 0.09)	0.252
Body iron, *mg/kg*	7.5 ± 2.3	7.4 ± 2.1	7.3 ± 2.2	7.9 ± 2.0	0.56[c] (0.29, 0.84)	0.18 (−0.09, 0.45)	0.956
R, *μmol/L*	1.04 ± 0.24	1.12 ± 0.24	1.08 ± 0.25	1.08 ± 0.23	0.041[a] (0.001, 0.08)	−0.011 (−0.05, 0.03)	0.060
DR/R, *mol/mol*	0.079 ± 0.05	0.06 ± 0.03	0.067 ± 0.05	0.058 ± 0.03	−0.013[c] (−0.02, −0.006)	−0.007 (−0.014, 0.00)	0.209
Plasma zinc, *μmol/L*	8.8 ± 1.9	9.4 ± 1.9	8.7 ± 1.9	9.4 ± 1.9	0.61[b] (0.26, 0.95)	−0.048 (−0.4, 0.30)	0.726
Urinary iodine,[3,4] *μg/L*	126 (83.8, 166.3)	165 (101, 217)	145 (87, 182)	168 (121, 210)	22.49[b] (7.68, 37.31)	11.75 (−3.04, 26.54)	0.701

[1] Values are means ± SD unless otherwise noted.
[2] Geometric mean (25th, 75th percentiles).
[3] Median (25th, 75th percentiles).
[4] To convert to μmol/L, multiply by 0.00788.
[5] Adjusted for age, baseline outcome values, and CRP levels (ANCOVA): [a] $P < 0.05$; [b] $P < 0.01$; [c] $P < 0.001$.

Both the deworming as the fortified biscuits improved micronutrient status, as can be seen in the table above, although the effect of the fortified biscuits was much higher than the effect of the deworming, and the impact of deworming failed to reach statistical significance. Interestingly, there was no interaction between deworming and the provision of fortified biscuits, meaning that the effect of the deworming is independent of the effect of the fortified biscuits. The only exception was for retinol concentrations. Indeed, we later showed that vitamin A is especially affected by ascaris infection, whereas iron status is mainly affected by hookworm infection (De Gier, AJTMH, in press). However, the interventions also had an impact on functional outcomes. The fortified biscuits had a significant impact on mid upper arm circumference (MUAC, +0.8 mm), but deworming also had a beneficial effect on MUAC (+0.7 mm).

TABLE 3
Anthropometric outcomes of the school children before and after four months of the intervention*

Outcome	Placebo (n = 122)	Fortified biscuits (n = 118)	Albendazole (n = 120)	Fortified biscuits plus albendazole (n = 122)	Estimated effect sizes† (95% CI), fortified biscuits plus albendazole		*P* for interaction fortification × deworming†
WAZ-scores							
Baseline	−1.56 ± 0.68	−1.52 ± 0.74	−1.47 ± 0.72	−1.55 ± 0.68	0.02	0.01	0.667
Endpoint‡	−1.48 ± 0.70	−1.42 ± 0.71	−1.37 ± 0.73	−1.44 ± 0.62	−0.01 to 0.05	−0.02 to 0.04	
HAZ-scores							
Baseline	−1.41 ± 0.86	1.47 ± 0.78	−1.40 ± 0.82	−1.44 ± 0.86	0.01	0.01	0.649
Endpoint‡	−1.34 ± 0.85	−1.39 ± 0.79	−1.33 ± 0.81	−1.36 ± 0.85	−0.001 to 0.03	−0.01 to 0.02	
WHZ-scores							
Baseline	−0.94 ± 0.72	−0.82 ± 0.78	−0.79 ± 0.81	−0.88 ± 0.78	0.03	0.01	0.626
Endpoint§	−0.86 ± 0.72	−0.72 ± 0.76	−0.70 ± 0.80	−0.78 ± 0.76	−0.02 to 0.08	−0.04 to 0.06	
MUAC (cm)							
Baseline	15.0 ± 1.1	15.1 ± 1.1	15.2 ± 1.2	15.0 ± 1.1	0.082¶	0.072¶	0.884
End point	15.3 ± 1.2	15.4 ± 1.1	15.6 ± 1.2	15.5 ± 1.1	0.02–0.15	0.01–0.13	

*Values are mean ± SD. Placebo = placebo de-worming and non-fortified biscuits; CI = confidence interval; WAZ = weight for age Z score; HAZ = height for age Z score; MUAC = mid upper arm circumference.
†Adjusted for sex, age, baseline outcome values, and C-reactive protein levels.
‡ $P < 0.001$, by paired *t*-test.
§ $P < 0.01$, by paired *t*-test.
¶ $P < 0.05$, by analysis of covariance.

The impact on other anthropometric indices were all positive, but failed to reach statistical significance. Perhaps the most significant finding was that the intervention improved cognitive scores in the children. Children receiving fortified biscuits, alone or with deworming scored higher on two out of 5 cognitive tests used. Interestingly, the effect of the fortified biscuits on the Raven's colored matrices test scores was higher in children anemic at baseline, pointing towards iron deficiency as a causative factor in lower cognitive scores in anemic children.

TABLE 4

Cognitive outcomes (in raw scores) of the school children before and after four months of the intervention*

Outcome	Placebo (n = 119)	Fortified biscuits (n = 114)	Albendazole (n = 118)	Fortified biscuits plus albendazole (n = 118)	Estimated effect sizes† (95% CI), Fortified biscuits plus albendazole		P for interaction
Raven's Colored Matrices							
All children							
Baseline	16.4 ± 5.6	16.5 ± 5.0	16.4 ± 5.3	16.5 ± 4.9	0.86	–0.18	0.486
End point	19.2 ± 5.8	20.1 ± 4.9	19.2 ± 5.4	19.4 ± 5.0	0.06–1.7	–0.97 to 0.62	
Anemic children‡							
Baseline	14.9 ± 5.1	15.6 ± 3.8	15.3 ± 5.6	15.4 ± 4.3	1.86§	0.10	0.971
End point	18.0 ± 5.1	20.6 ± 4.4	18.2 ± 5.8	20.8 ± 4.2	0.46–3.3	–1.29 to 1.50	
Digit span forward							
Baseline	7.1 ± 1.4	6.9 ± 1.2	6.9 ± 1.5	7.1 ± 1.2	0.34¶	0.07	0.310
End point	7.1 ± 1.4	7.5 ± 1.1	7.1 ± 1.3	7.4 ± 1.1	0.11–0.56	–0.15 to 0.30	
Digit span backward							
Baseline	2.9 ± 0.9	2.9 ± 1.0	2.8 ± 1.0	2.9 ± 0.8	–0.07	–0.03	0.701
End point	3.1 ± 0.8	3.1 ± 0.9	3.1 ± 0.8	3.0 ± 0.9	–0.25 to 0.10	–0.21 to 0.14	
Block design							
Baseline	11.8 ± 8.5	12.5 ± 8.7	11.1 ± 8.4	12.8 ± 7.4	–1.12	0.42	0.726
End point	16.6 ± 9.0	16.5 ± 8.6	16.9 ± 9.6	17.0 ± 9.1	–2.48 to 0.23	–0.94 to 1.77	
Coding							
Baseline	31.9 ± 10.0	30.8 ± 8.4	32.2 ± 9.4	30.8 ± 8.6	0.50	0.54	0.603
End point	39.6 ± 8.0	39.5 ± 7.0	39.6 ± 7.8	39.5 ± 8.5	–0.95 to 1.94	–0. 90 to 1.99	

* Values are mean ± SD. Placebo = placebo de-worming and non-fortified biscuits; CI = confidence interval. There were no significant differences between groups at baseline.
† Adjusted for age, family socioeconomic status, mother's education, and baseline outcome values.
‡ Sample sizes for placebo, fortified biscuits, albendazole, and fortified biscuits plus albendazole were 29, 30, 28, and 31, respectively.
§ $P < 0.05$, by analysis of covariance.
¶ $P < 0.01$, by analysis of covariance.

Overall, the study also showed that combining deworming with food fortification can have additional benefits on both micronutrient status as on functional outcomes even though the 2 interventions are independent of each other.

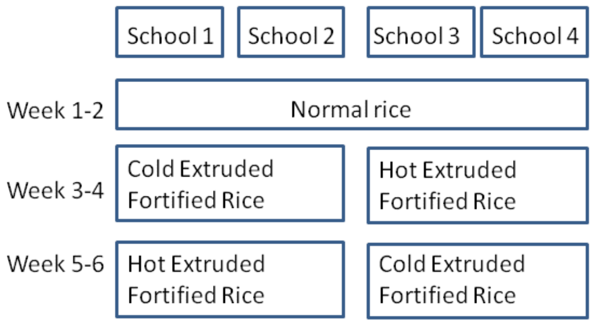
4.4.3. Rice fortified with multiple vitamins and minerals: acceptability.

In South-East Asia, rice is a highly regarded food, and changes in taste, color or smell are likely to impact consumption significantly. Therefore, before fortified rice can be introduced on a large scale in e.g. Vietnam or Cambodia fortified rice needs to be found acceptable with regard to organoleptic qualities. To underline the scale of the potential of fortified rice: in Cambodia the World Food Program (WFP) provides >500.000 school children with a daily school meal (breakfast), consisting of rice and beans.

In 2010, we conducted a 2 trials on the acceptability of fortified rice; one in Vietnam and one in Cambodia.

In Vietnam, the main objective of the study was to assess the attitudes of end-consumers (women of reproductive age) and village rice millers to the use and taste of fortified rice. For this purpose, women of reproductive age (n=430) and rice millers in 2 villages in Bac Giang province, North-East Vietnam were invited to participate in the study. Two types of fortified rice (hot and cold extruded) were used in the study. Both types of fortified rice ('rice premix') were mix with normal rice ("khang dang"). Fifty-three women at reproductive age were asked to participate in a triangle test with fortified and normal rice. Each woman received 3 coded bowls of rice, of which two bows are similar (either normal or fortified rice). In target groups, women were interviewed and asked to score on a 9 points scale from extremely don't like to extremely like the fortified rice and the normal rice.

The study in Cambodia was a combined sensory testing and consumption trial in primary school children (n=1700) receiving a school meal through the WFP school meal program, and their parents and teachers. The consumption trial was a single blinded, cross-over, placebo controlled trial, in



which each school received 2 weeks of each type of fortified rice. All schools started with 2 weeks of normal rice as run-in. Thereafter, two schools first received 2 weeks of cold extruded fortified rice, followed by 2 weeks of hot extruded fortified rice whereas in the other 2 schools the sequence was the opposite (Figure). Teachers and parents were invited for testing the fortified rice for organoleptic qualities. Teachers and parents first participated in a Triangle Test (see above), whereafter, they were asked to score organoleptic qualities of normal and cold-extruded fortified rice on a scale of 1 (bad) to 5 (excellent) for taste, smell, appearances and color. Each second week, 200 students (50 from each school) selected at random from class 4 – 6 were also asked about the organoleptic qualities of the rice consumed during the week.

During the triangle test, most of the women (>75%) in Vietnam and Cambodia as well as the Cambodian teachers (>62%) correctly identified the bowl with fortified rice. In Vietnam, <5% of the women indicated that they didn't like the fortified rice. Smell and taste were scored higher for the fortified rice than for the normal rice. In contrast, the Vietnamese women indicated that white rice was preferred over colored rice, even though the color indicated fortification. In Cambodia, parents and teachers scored the fortified rice better for taste (P<0.001) and smell (P=0.025) than normal rice, with similar organoleptic qualities for color and appearance. School children (n=200) scored both cold and hot extruded rice significantly higher than normal rice for taste and smell (P<0.001 for both), but with no differences for color, appearance or mouth feel (stickiness and hardness). The higher acceptability of fortified rice compared to normal rice also translated to a higher consumption of rice during the acceptability trial in Cambodia. The mean amount of cooked normal rice eaten by each child during the first two weeks of the trial was 166 g (± 58 g) of rice/meal/day. Introduction of fortified rice, regardless of type (cold or hot extruded), resulted in an overall significant increase in rice consumption, with on average 176 g (± 49 g) of fortified rice consumed per child per meal, an increase of 10 g of rice per child per day. However, there was a significant interaction between school and change in amount of rice consumed by the children. In 2 schools rice consumed per child increased (with 39 and 34 g of rice per child respectively, P<0.001), whereas in the other 2 schools, rice consumption remained the same. Hence, we shpwed that the introduction of fortified rice is feasible in SE Asia with regard organoleptic qualities of fortified rice. Introduction of fortified rice through large food assistance or food-based social safety net programs, such as the WFP School Meals Program, can be relatively straightforward, which normal rice being blended with fortified rice kernels at a central location. In contrast, fortification of rice at village level poses many possible barriers, including assuring the correct blending ratio and obtaining homogenous mixtures without too much variation.

4.4.4. Stability of micronutrients in fortified rice during storage and cooking.

Unfortunately, even though rice fortified with minerals and vitamins is acceptable for consumers in South-East Asia, not all vitamins are stable during storage or cooking, as we showed in 2 different studies. Especially vitamin A is very sensitive to cooking and storage, with losses of >90% for some types of fortified rice.

Table 4. Retention of vitamin A in cooked rice prepared in five different ways, as percentage from vitamin A concentration in uncooked rice, for the six different producers of fortified rice

	Methods					
Vitamin A	Excess + soaking	Excess	Boiling	Boiling + washing	Frying	Overall
CO1	91.8 (±1.2)^{a}	5.1 (±4.4)^{b,1}	14.3 (±16.5)^{b,1}	57.9 (±12.2)^{c,1}	33.7 (±12.3)^{b,c}	40.6 (±33.8)^{1,3}
CO2	92.3 (±21.5)^{a}	0.0 (±0.0)^{b,1}	4.9 (±0.7)^{b,1}	54.9 (±22.6)^{a,c,1}	20.6 (±5.0)^{b,c}	34.5 (±37.8)^{1,3}
CE1	67.6 (±10.7)^{a}	6.3 (±10.9)^{b,1}	20.9 (±23.9)^{a,b,1}	59.8 (±13.8)^{a,b,1}	34.5 (±30.9)^{a,b}	37.8 (±29.1)^{1,3}
CE2	79.8 (±3.3)	82.1 (±9.1)^{2}	86.5 (±3.3)^{2}	75.1 (±9.1)^{1}	70.5 (±5.8)	78.8 (±8.0)^{2}
HE1	71.3(±3.0)^{a}	3.9 (±6.8)^{b,1}	13.7 (±13.4)^{b,c,1}	14.1 (±1.3)^{b,2}	30.0 (±19.7)^{b}	26.6 (±26.4)^{1}
HE2	85.9 (±8.0)^{a}	2.2 (±3.9)^{b,1}	24.7 (±23.2)^{b,c,1}	61.7 (±14.4)^{a,c,1}	36.1 (±11.6)^{b,c}	42.1 (±32.3)^{3}
Overall	81.5 (±13.2)^{a}	16.6 (±30.8)^{b}	27.5 (±31.0)^{b,d}	53.9 (±22.6)^{c}	37.6 (±21.3)^{d}	43.4 (±33.2)

NOTE: Rows with different letters are significantly different from each other ($P < 0.05$).
Columns with different numbers are significantly different from each other ($P < 0.05$).
$P_{interaction}$ method × producer <0.001.

From Wieringa et al, NYAS (2014)[59]

In contrast, iron and zinc were far more stable over time and during cooking, with retentions in the range from 80-100%.

Table 3. Retention of iron in cooked rice prepared in five different ways, as percentage from iron concentration in uncooked rice, for the six different producers of fortified rice

	Methods					
Iron	Excess + soaking	Excess	Boiling	Boiling + washing	Frying	Overall
CO1	89.7 (±3.4)^{1}	87.4 (±27.1)	90.9 (±8.6)^{1}	87.7 (±5.9)^{1}	82.3 (±34.6)	87.6 (±17.4)^{1}
CO2	98.6 (±32.5)^{1}	126.3 (±70.2)	89.5 (±6.5)	93.0 (±21.2)^{1}	69.8 (±10.0)	95.4 (±36.0)^{1}
CE1	79.1 (±12.3)^{1}	96.2 (±63.6)	107.2 (±11.2)^{1}	97.8 (±4.3)^{1}	93.3 (±6.0)	94.7 (±26.7)^{1}
CE2	209.6 (±20.6)^{a,2}	154.7 (±19.5)^{b}	208.4 (±11.3)^{a,2}	94.9(±15.5)^{c,1}	107.6 (±10.8)^{c}	155.0 (±51.9)^{2}
HE1	64.7 (±15.0)^{1}	58.1 (±9.1)	85.2 (±19.4)^{1}	46.0 (±4.0)2	83.4 (±16.6)	67.5 (±19.5)^{1}
HE2	97.4 (±13.8)^{1}	86.1 (±27.8)	107.7 (±9.2)^{1}	88.2 (±4.8)^{1}	114.6 (±74.5)	98.8 (±32.8)^{1}
Overall	106.5 (±51.3)^{a,b}	101.5 (±48.1)^{a,b}	114.8 (±45.1)^{a}	84.6(±20.5)^{b}	91.8 (±33.3)^{a,b}	99.8 (±41.7)

Note: Rows with different letters are significantly different from each other ($P < 0.05$).
Columns with different numbers are significantly different from each other ($P < 0.05$).
$P_{interaction}$ method × producer <0.001.

Storage of fortified rice also resulted in a quick disappearance of vitamin A, whereas iron and zinc were retained. There were big differences between the type of fortified rice, with extruded rice having significant higher retentions for vitamin A over time (70-78% at low temperatures, 40-50% at high temperatures after 1 year) than coated rice (23% and 7% at low and high temperatures respectively). Retention for iron and zinc were all >90% [60].

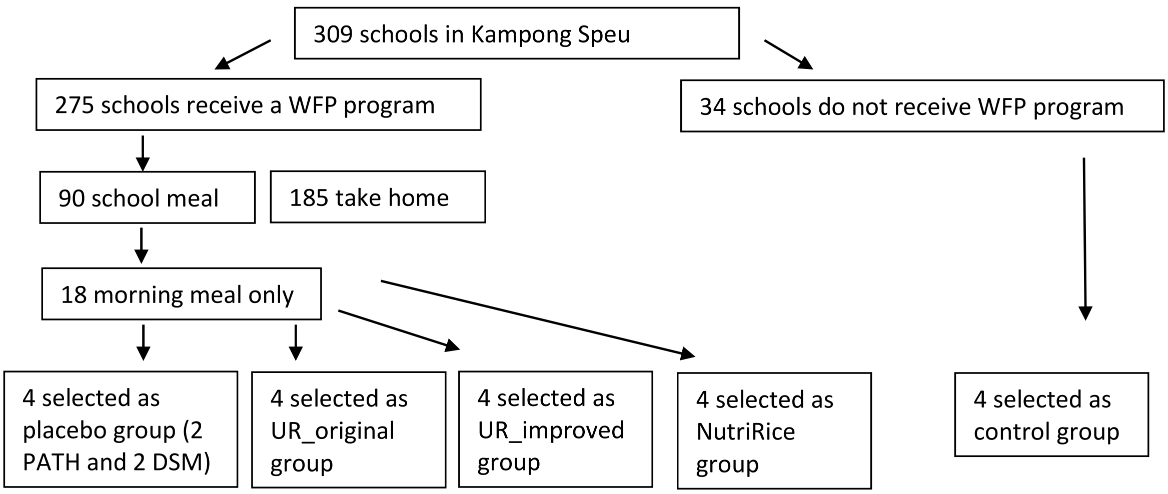
Table 2. Retention of retinyl palmitate over time, as percentage from retinyl palmitate concentration in uncooked fortified rice prior to storage (T0).

		25 °C/60% Humidity		
Type	**T0**	**T90**	**T180**	**T360**
Hot extrusion	100	103.1 (± 5.1) ^{c,5}	90.6 (± 7.0) ^{c}	78.9 (± 12.9) ^{c,1,3}
Cold extrusion	100	94.6 (± 12.1) ^{c,5}	83.4 (± 5.8) ^{c,1}	70.1 (± 8.0) ^{c,1,3}
Coated	100	77.5 (± 7.7) ^{a,b,1,4,5}	43.7 (± 14.2) ^{a,b,1,3,5}	23.1 (± 15.8) ^{a,b,1,3,4}
		40 °C/75% Humidity		
Type	**T0**	**T30**	**T90**	**T180**
Hot extrusion	100	78.7 (± 5.5) ^{c,1,4}	78.0 (± 13.7) ^{c,1,4}	51.5 (± 14.2) ^{c,1,2,3}
Cold extrusion	100	80.1 (± 2.3) ^{c,1,3,4}	64.7 (± 7.0) ^{c,1,2,4}	39.3 (± 5.7) ^{c,1,2,3}
Coated	100	40.6 (± 15.2) ^{a,b,1,3,4}	17.6 (± 14.1) ^{a,b,1,2}	6.9 (± 7.8) ^{a,b,1,2}

Note: All values are means ± SD. ^{1} significantly different from T0; ^{2} significantly different from T30; ^{3} significantly different from T90; ^{4} significantly different from T180; ^{5} significantly different from T360; ^{a} significantly different from Hot extrusion; ^{b} significantly different from Cold extrusion and ^{c} significantly different from Coated.

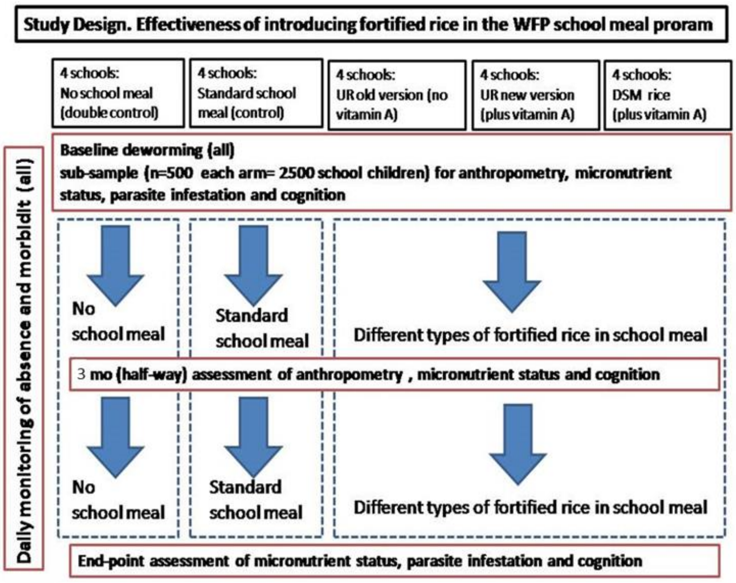
4.4.5. Fortified rice for school children in Cambodia (FORISCA): Impact on micronutrient status.

Although we had shown that fortified rice was acceptable in SE Asia, and although rice fortification has been shown to be safe and effective in improving micronutrient status in many studies, no studies available from Cambodia. Therefore, it was unknown whether fortified rice could reduce the prevalence of anemia in school children in Cambodia, nor whether there were additional benefits, such as better learning capability of school children or fewer days missed due to better health, or improved growth and anthropometrical status. To inform the World Food Program and Cambodian policy makers on the potential benefits of fortified rice for health and development in Cambodian school children, a large intervention study was conducted in 16 schools in Kampong Speu province in Cambodia. Schools were part of the standard WFP school meal program. Schools were randomly allocated to receive either the normal rice provided by the WFP school meal program (4 schools = placebo), or one of 3 types of fortified rice instead of the normal rice (12 intervention schools). The 3 different types of fortified rice differed in terms of production (hot or warm extruded) and vitamin/mineral content. In addition, 4 schools from the same province with no WFP school meal program were randomly selected to serve as control.



Randomization of the schools in Kampong Speu for the FORISCA trial

School attendance and morbidity were monitored in all children (N=9500) over the whole school year, whereas biochemical indicators of micronutrient status, intestinal parasite infestation, anthropometry and cognitive tests scores were obtained from a subgroup of children (2500 children, 25%) at baseline, midline and endpoint. For each school, a random list of 168 eligible children for the in-depth study was made. The list of eligible children was stratified for sex and grade (14 boys and 14 girls for each of the 6 grades, making 168 children in total).



Another objective of the FORISCA study was to compare three different types of fortified rice. There are several methods for producing fortified rice. Two widely used methods are extrusion and waxing. With the extrusion method, artificial rice kernels are made from a mixture of rice flour, a premix of vitamins and minerals, and a binding agent. Extrusion can be cold (temperaturs <70°C), warm (between 70°C and 100°C) or hot (temperatures >100°C). The artificial rice kernels are mixed at a ratio of 1:100 with normal rice, making fortified rice suitable for consumption. During the FORISCA trial, a cold (UR_original), a warm (UR_improved) and a hot (NutriRice) extruded fortified rice kernel were evaluated.

Comparison Fortified Rice Kernels Premix Forisca Study

MICRONUTRIENT	TARGET VALUE (mg)	NUTRIRICE	ULTRA_ORG	ULTRA_IMP
VITAMIN A	0,3	0,288		0,64273
IRON	7,26	7,46	10,67	7,55
ZINC	3,5	3,68	3,04	2,02
VITAMIN B1 (Thiamine)	0,6	0,688	1,06	1,43
VITAMIN B3	8	7,98		12,57
VITAMIN B6	0,65	0,92		0
FOLIC ACID	0,2	0,135	0,172	0,278
VITAMIN B12	0,0012	0,00126		0,0038

Unfortunately, due to an error in using the correct premix (UltraRice_Original), and due to changes made to the premix prior to final production by PATH (UltraRice_Improved), the micronutrient levels were not the same in the 3 different kernels, with only NutriRice providing rice premix kernels that fulfilled the pre-set levels for all micronutrients. These differences between the 3 types of rice kernels make comparison of the biochemical and functional outcomes far more difficult.

The overall nutritional status of the children could be characterized as 'moderately malnourished', with a mean BMI-for-Age Z-score (BAZ) of -1.51, and a mean Height-for-Age Z-score (HAZ) of -1.80. Anemia prevalence was ~17% in the the school children in the intervention groups and ~10% in the control schools. In general, the children in the control schools had a better nutritional status and a higher social-economic status, one of the reasons these schools were not selected by WFP for the school meal program.

Risk factors for being anemic included hemoglobinopathy, depleted iron stores, vitamin A deficiency and parasite infection[61]. The prevalence of abnormal forms of hemoglobin (hemoglobin forms other than HbA) was high, with 49% of the children having some form of hemoglobinopathy. HbE was the most prevalent form of hemoglobinopathy, with especially homozygous HbE (8% of the school children) carrying a very high risk of anemia[61].

After 3 months, the intervention had a significant effect on hemoglobin concentrations and iron status when compared with placebo group (interaction effect: $p<0.001$ for all) with higher (+0.8 g/L) hemoglobin concentrations in children receiving UltraRice_improved as compared with children receiving unfortified rice ($p<0.05=0.048$). However, after 6 months of intervention, there were no significant differences among the groups anymore with regard to hemoglobin concentrations.

Inflammation status was a significant effect modifier of the intervention on hemoglobin concentrations however. In the school children, the prevalence of subclinical inflammation was high, as indicated by elevated concentrations of 2 acute phase proteins: C-reactive Protein (CRP) and α1-acid-glycoprotein (AGP). At baseline, around 42% of the school children had some form of inflammation. This percentage dropped to around 25% at midline, and 31% at endline. For children with no inflammation (both CRP<5mg/L and AGP <1g/L) at baseline, midline and endline, hemoglobin concentrations significantly increased by 2.1 g/L after 3 months in UltraRice_improved group when compared with placebo ($p<0.01$). The increase was still significant after 6 months in this group (+1.88 g/L, $p=0.015$). Hemoglobin concentrations also tended to be higher after 6 months in the two other groups receiving fortified rice: UltraRice_original and Nutririce ($p=0.054$ and $p=0.095$ respectively) for this sub-sample of children with no inflammation.

Another sub-group of children which appears to benefit from fortified rice in terms of improvements in hemoglobin concentrations are children with a low iron status at baseline. In children with total body iron (a composite indicator, using ferritin and sTfR concentrations) of <4 mg/kg body weight, consumption of UR_improved rice resulted in a tendency towards higher hemoglobin concentrations as compared to control (4.1 g/L higher; P=0.06).

Vitamin A status, as indicated by serum Retinol-Binding-Protein (the protein transporting vitamin A in the circulation), was relatively good at baseline, with only a small percentage (<1%) of children being vitamin A deficient (RBP<0.70 μmol/L), and another ~8% of the children having marginal vitamin A status (RBP between 0.70 and 1.05 μmol/l). Interestingly, the prevalence of marginal vitamin A status increased in all groups receiving normal rice or fortified rice not containing vitamin A, and declined in the groups receiving fortified rice with vitamin A (UR_improved and NutriRice). As a result, consumption of NutriRice and UltraRice significantly reduced the risk of having marginal vitamin A status by 76% and 80% respectively when compared with the placebo group (P<0.001). Vitamin A status was an important predictor of anemia too, with the prevalence of anemia almost twice higher for children with marginal or deficient vitamin A status (corrected RBP<1.05 μmol/L) than in children with normal VA levels (24.5% vs. 15.0%).

The concentrations of serum zinc were low, and hence the prevalence of zinc deficiency was very high, at all time-points. Indeed, for all groups for all time-points, the prevalence of zinc deficiency (>70%) far exceeded the threshold of 20% deficiency set by WHO as a health problem. After the 6 months intervention, zinc concentrations in all fortified rice arms (UR_original and UR_improved) were significantly higher as compared to placebo rice (ANCOVA, controlling for baseline zinc concentration, age, gender). Subsequently, the prevalence of zinc deficiency declined significantly over the intervention in all groups receiving fortified rice

When examining the changes over the intervention period, children consuming rice fortified with zinc had much lower adjusted OR's for being zinc deficient ranging from a OR of 0.19 for NutriRice to 0.43 for UR_improved, as compared to children consuming placebo rice.

In a sub-group of samples, serum folic acid concentrations were measured also. Results showed that in children receiving rice fortified with folic acid, serum concentrations were significantly higher.

Hence, overall, FORISCA showed that fortified rice improved vitamin A, zinc and folic acid status, but had little or no impact on iron status or hemoglobin concentrations. In the following chapter, effects on functional outcomes such as cognitive function, morbidity and growth will be discussed.

5. Interactions between micronutrients

5.1. Introduction

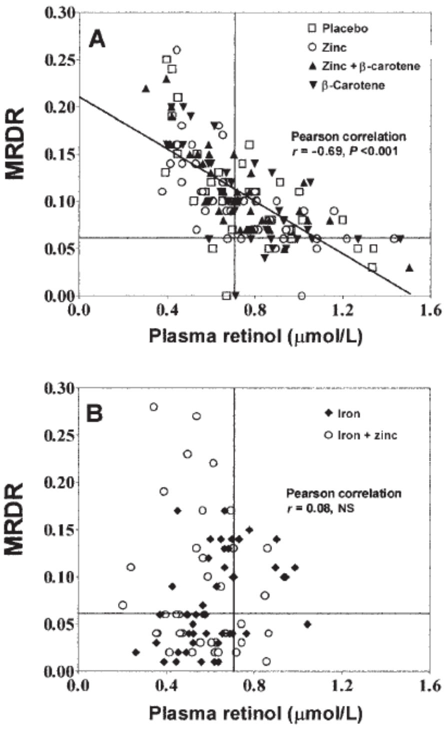
With the increasing understanding of human health, metabolism and growth over the last decades, many more distinct functions of many micronutrients has become clear. This led to a growing appreciation of the complexity micronutrient metabolism and of the many interacting pathways of micronutrients. However, when we started working on vitamin A, iron and zinc in Indonesia in the mid 1990's, interactions between micronutrients were seldom taken into account, despite evidence of for example a beneficial effect of vitamin A when added to iron supplements[62]. Instead, interactions among micronutrients are often disregarded for the sake of simplicity, and because they are often difficult to study or quantify. Interactions may occur at different levels, such as in the food itself (food matrix), during absorption from the gut (competition for receptors) or once absorbed in the body (metabolic interactions). Interactions can be both beneficial as harmful. For example, iron bioavailability is enhanced by concurrent consumption of vitamin C, as vitamin C reduces $Fe3+$ to the more soluble $Fe2+$. But bioavailability of both iron and zinc is reduced by high calcium intake. The effects of interactions between micronutrients can be exacerbated when micronutrients are supplemented in relatively high doses, and in situations where micronutrient status is already marginal or deficient.

During my research on micronutrient interactions, I focused mainly on interactions between vitamin A, iron and zinc. Iron supplementation was shown to be less effective in improving anemia in the presence of vitamin A deficiency, and as vitamin A deficiency was prevalent in Indonesian infants, it was logical to take this interaction into account during our studies in Indonesia. The effect of vitamin A on iron metabolism appears to take place either in the bone marrow (involving erythropoietin) and/or through increasing the mobilization of iron for hematopoiesis[63], although the exact roles and interactions of vitamin A in iron metabolism have still not been fully elucidated. However, multiple micronutrient interventions do not necessarily yield simple additive or synergistic benefits as antagonist effects are quite common too, sometimes nullifying the hoped-for health benefits.

The effect of vitamin A on iron metabolism appears on the first hand to be beneficial (higher efficacy), but it has a downside too. Vitamin A supplementation without providing additional iron can decrease iron status, by forcing the utilization of iron for hemoglobin synthesis. Although we saw evidence for this in our study in Indonesian infants, with infants who received zinc + β-carotene having a significant higher prevalence of iron-deficiency anemia than infants who received placebo[10], but we lacked the statistical power to attribute this effect to vitamin A or b-carotene. The South-East Asia Multi-country trial on Iron and Zinc supplementation in Infants (SEAMTIZI) gave us enough statistical power to show that indeed a high dose of vitamin A given to infants with a marginal iron status might increase the prevalence of iron deficiency[16]. Although the SEAMTIZI trial was not designed to investigate the effect of vitamin A on hemoglobin concentrations, high-dose vitamin A capsule distribution to the children was different among the study sites, allowing us to look at the effect of the standard high-dose vitamin A capsule program on iron status. Whereas vitamin A given prior to the study tended to contributed towards higher efficacy of the iron supplementation, leading to higher hemoglobin concentrations, the opposite was true for infants not receiving iron, with hemoglobin concentrations lower in infants receiving vitamin A as compared to infants receiving nothing. This is cause for concern because high-dose vitamin A capsule distribution is given to over 75% of all children <5 yrs of age living in least-developed countries, or over 500 million Vitamin A capsules per year. Iron deficiency in infancy and childhood has been linked to impaired cognitive development, sometimes with permanent effects. If high-dose vitamin A capsules indeed renders infants and young children more susceptible for iron deficiency, than this is a very serious side-effect of the global high-dose vitamin A capsule programs. In 2007, we urged for more research into this interaction, but to my knowledge, this has not been undertaken until today.

The studies in Indonesia and the large SEAMTIZI trial proved to be very valuable to explore other micronutrient interactions also. At that time, the interactions between iron and zinc were considered minor and not important in public health nutrition respect[64]. We showed with the SEAMTIZI data that the combination of iron and zinc was less effective in reducing the prevalence of anemia than iron alone (21% and 28% reduction respectively)[16]. And vice-versa, iron and zinc combined was less effective in reducing the prevalence of zinc deficiency (reduction of 10%) than zinc alone (18% reduction). Interestingly, the effect of iron supplementation on hemoglobin concentrations was almost twice as large in boys as in girls (+12.0 vs. +6.8 g/L, respectively). We surmise that the stronger physical growth of boy infants in the first year of life renders them more susceptible for iron deficiency, and indeed increases their iron requirements as compared to girl infants, with requirements being 0.9 mg/d higher[15].

But, to complicate matters, provision of iron to infants might affect vitamin A status, as also shown by the studies from Indonesia, in which the supplementation of iron caused a redistribution of vitamin A from the circulation to the liver (storage)[10]. In the trial in Indonesia, infants received supplements (iron and/or zinc and/or b-carotene) for 6 months. At the end of the trial, infants who had received



iron alone or in combination with zinc had a much higher prevalence of vitamin A deficiency when measured through plasma vitamin A concentrations (70-75% of deficiency compared to 53% in placebo group). Yet at the same time, an indicator for vitamin A liver stores, the Modified Relative Dose Response (MRDR) test, showed that the infants who had received iron (alone or combined with zinc) had much better vitamin A liver stores. This is depicted in the 2 graphs above. In the lower graph, almost half of the infants who have received iron are in the left-lower quadrant, which depicts deficient circulating vitamin A concentrations (plasma retinol < 0.70 mmol/l) combined with adequate vitamin A liver stores (MRDR <0.05). There is no correlation in these children between vitamin A stores and circulating vitamin A concentrations. In contrast, in children receiving no iron (graph on top), there is a very strong correlation between vitamin A stores and circulating vitamin A concentrations, with circulating vitamin A concentrations becoming higher with higher vitamin A liver stores, as one would expect.

But not all the micronutrient interactions we found were negative. Again from research from Indonesia, we showed that the addition of zinc and β-carotene to standard iron and folic acid supplements for pregnant women improved not only the vitamin A status of the women, but also of the newborns six months after delivery[5] presumably by enhancing the conversion of b-carotene to retinol through some yet unknown mechanism. Interestingly, the addition of only b-carotene to the iron-folic acid supplements did increase b-carotene concentrations in breast-milk, but did not increase retinol concentrations, really underscoring the importance of zinc in this context[5].

TABLE 3
Micronutrient concentrations in breast-milk samples at 1 and 6 mo postpartum

	Supplementation group			
Breast-milk concentration	Control (n = 31)	β-Carotene (n = 35)	Zinc (n = 29)	β-Carotene + zinc (n = 29)
Fat (g/L)				
1 mo	30.1 ± 12.0[1]	34.4 ± 12.2	33.9 ± 15.8	31.6 ± 9.6
6 mo	33.8 ± 14.0	32.5 ± 16.2	34.2 ± 16.5	30.5 ± 14.2
Retinol (nmol/g fat)[2,3]				
1 mo	50.6 (44.1–88.6)[4]	32.7 (34.4–73.5)	51.3 (41.4–80.0)	54.0 (30.9–105.6)
6 mo	27.9 (18.9–36.6)	30.9 (19.7–47.1)	30.4 (19.8–48.0)	40.7 (25.1–57.0)[5]
β-Carotene (nmol/g fat)[3]				
1 mo	0.59 (0.32–1.18)	0.81 (0.49–1.16)	0.49 (0.29–1.17)	0.54 (0.40–1.31)
6 mo	0.56 (0.34–0.71)	0.89 (0.53–1.38)[1]	0.73 (0.48–0.90)	0.80 (0.62–1.32)[6]
Zinc (μmol/L)[2,3]				
1 mo	42.1 (31.1–51.7)	42.1 (33.0–62.6)	49.3 (31.3–62.1)	46.7 (37.0–61.8)
6 mo	16.8 (11.2–24.3)	17.7 (11.8–29.2)	15.3 (11.8–27.2)	17.7 (12.9–27.4)

[1] $\bar{x}$ ± SD (all such values).
[2] Significant decrease from 1 to 6 mo, $P < 0.001$ (independent t test, with all groups pooled).
[3] Transformed to natural logarithms before statistical analysis.
[4] Median; interquartile range in parentheses (all such values).
[5,6] Significantly different from control group (analysis of covariance followed by Tukey's post hoc test): [5] $P < 0.05$, [6] $P < 0.01$.

The above mentioned interactions were all on biochemical level, either through interactions during absorption or due to metabolic interactions. However, we also found interactions on functional level. For example, in the SEAMTIZI trial, zinc supplements only benefitted infants in terms of length growth when they were anemic at baseline (+0.17 Z-score), whereas there was no effect in infants not anemic at baseline (-0.04 Z-score)[47]. Moreover, the infants anemic at baseline receiving only zinc benefitted more in terms of linear growth than children receiving the combination of iron + zinc. Anemia is of course a poor indicator of iron status, as anemia can be due to other micronutrient deficiencies or to non-nutritional causes. But one would expect iron to be beneficial in infants with anemia at baseline. The effects of iron supplementation on anthropometric indices were not statistical significant (HAZ +0.02 ; WHZ +0.06) however. The difference between iron and zinc in physiological terms is of course that iron is a classic, Type-1, nutrient not directly implicated in growth, whereas zinc is a typical Type-2 or growth nutrient[65]. Hence, effects of growth are more likely to become apparent with zinc than with iron. There was a negative effect of iron on linear growth however, namely in infants with a birth weight >3500 g, with an estimated -0.14 Z score in infants receiving iron for 6 months. This negative effect of iron on linear growth has been reported before, and has been linked to iron status. That is, iron supplements negatively affect linear growth in children with adequate iron status, but are beneficial for children who are iron deficient[66].

Interestingly, there appears to be an effect of the vitamin A containing rice on iron status also, as the increase in ferritin concentrations was found only in the UltraRice_improved and Nutririce groups. Indeed, although UltraRice_original contained the highest concentration of iron, there was no increase in iron stores, whereas the highest increase in FER was found in the rice with the highest vitamin A content (UltraRice_improved). Vitamin A has been shown to increase iron mobilization from stores and to improve erythropoiesis. However, it appears that in the present study, erythropoiesis was increased without mobilization of additional iron from stores, given the higher TfR concentrations in the 2 vitamin A containing fortified rice groups.

6 Micronutrients and functional outcomes: cognitive function, morbidity and immunity

6.1 Introduction

Although improvements in micronutrient status after interventions, being supplementation or food-based approaches, are welcome from a biochemical point of view, in the end, one hopes to improve functional outcomes, as the final objective is to improve overall health and well-being of the subject or population. Functional outcomes can be very diverse, ranging from improved growth ('optimal growth') to better cognitive performance to better immunocompetence with a reduction in morbidity and mortality.

In this chapter, I will review the impact of several of the interventions I have been involved in on functional outcomes, starting with perhaps the most obvious outcome: growth. Thereafter, I will discuss effects on the immune system and finally on cognitive development.

6.2. Effects of micronutrient interventions on the immune system

6.2.1. Introduction

The immune system is a complex, not completely understood organ. Simply speaking, it consists of a non-specific or innate component, forming a constant defense mechanism that does not adapt to an invading agent, and a specific immune defense, that is capable of responding in an antigen-specific way. The most basic innate defense mechanisms include integrity of the epithelial surface and mucosal barrier function. Humoral components of non-specific immunity include opsonins, complement activation and the acute phase response. Non-specific, cell-mediated immunity is formed by macrophages and other phagocytic cells. Innate immunity is not improved by repeated exposure to infectious agents.

Lymphocytes are central to the specific or adaptive immune response. T-lymphocytes (T because these cells mature in the thymus) are essential in the regulation of immune responses, and can be divided into different subpopulations according to their function, or rather according to specific markers. Main classes of T-cell that can be distinguished are the cytotoxic T-cells, which can kill target cells, and the T-helper cells, which can activate macrophages and B-lymphocytes. B-lymphocytes (B from Bursa Fabricius*,* an organ associated with the gut specific for birds where these cells were first identified) mature in the bone marrow, and can be induced to produce antibodies in response to specific antigens, and/or T-helper cell stimulation. Cytokines are produced by the cellular components of the immune system to specific stimuli, and play a key role in the modulation and regulation of immune function. Cytokines induce responses in a wide range of effector cells, and can initiate, stimulate and suppress immune reactivity.

The immune system of newborn infants is still immature, develops during the first year of life, reaching adequate immunocompetence at about one year of age. During the first few months of life, maternal antibodies (especially IgG), acquired *in utero*, still circulate and protect the newborn. During the first months of life, the immune responses are very much skewed towards so-called Th2 responses (humoral immunity), whereas Th1 responses (cellular immunity) only develops during the first year.

The important role of micronutrients in immune function has only emerged recently, although vitamin A has been known as the "anti-infective vitamin" since the 1920's. Vitamin A deficiency appears to affect both the innate and specific immune system, as vitamin A is necessary for cell differentiation, phagocytosis and the modulation of cytokines. Studies, mostly in animal models, have shown specific effects of vitamin A deficiency on the immune system, however often with conflicting results. A general depression of T-cell activation is reported by some studies, supported by findings of decreased interferon-γ (IFN-γ) production and decreased natural killer cell activity, as well as suppression of the delayed type hypersensitivity response. Other studies have found overproduction of IFN-γ and reduced antibody production, fitting with a Th1 predominance in vitamin A deficiency. Severe zinc deficiency is accompanied by a marked increase in susceptibility to infections, but the effects of marginal zinc status are less well documented. In population studies, zinc supplementation gave a marked reduction in morbidity of diarrheal diseases and respiratory infections. On a cellular level, zinc deficiency results in a striking depletion of both B- and T-cells, and also decreases numbers of neutrophilic granulocytes, natural killer cells and macrophages, as well as reduced cytokine production and antibody response. The role of iron in immune function has been the subject of much debate. On the one hand, iron is needed for various immunological functions, not in the least cytotoxicity and phagocytosis. Cellular functions such as cytotoxicity can already be reduced in marginal iron deficiency, before anemia is present. On the other hand, iron is essential for the proliferation of most bacteria. Thus iron deficiency not only impairs immunological functions, but also suppresses bacterial growth. To complicate matters, supplementation with iron can enhance immune reactivity to such an extent that damage arises from the exacerbated inflammatory response. In population studies, the effects of iron supplementation on immune function has not been consistent, with studies from malaria endemic areas reporting increases in morbidity and mortality prevalence [67].

6.2.2. Effects of micronutrient deficiency and micronutrient supplementation on immune function

We investigated the ability of circulating leukocytes to produce cytokines upon stimulation. Whole blood was incubated with strong immune stimuli (LPS, PHA) and incubated at 37°C for 24 hrs. This culture system can be regarded as a model for sepsis. Cytokines were measured in the supernatant. First of all, these experiments showed a clear effect of micronutrient deficiency in Indonesian infants on their ability to produce cytokines.

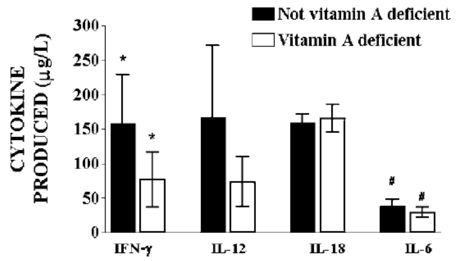


Figure 1 *Ex vivo* whole blood cytokine production after stimulation with LPS and PHA. Mean production (±95% CI), in vitamin A deficient infants ($n=25$) *vs* nonvitamin A-deficient infants ($n=27$). Differences between groups: *: $P<0.05$ (Student's *t*-test), #: $P<0.1$ (Mann–Whitney *U*-test). IFN-γ: interferon-γ; IL:interleukin.

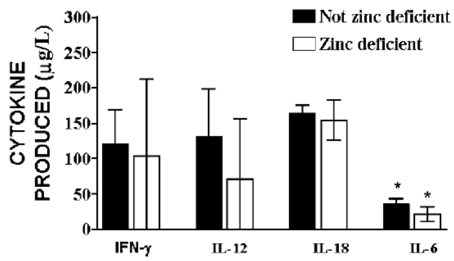
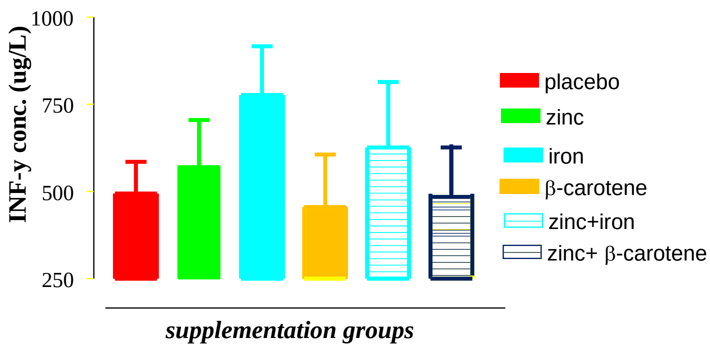


Figure 2 *Ex vivo* whole blood cytokine production after stimulation with LPS and PHA. Mean production (±95% CI), in zinc deficient infants ($n=9$) *vs* non-zinc-deficient infants ($n=43$). Differences between groups: *: $P<0.05$ (Mann–Whitney *U*-test). IFN-γ: interferon-γ; IL: interleukin.

But zinc, iron and vitamin A deficiency affected the immune system in different ways, with iron and vitamin A deficiency leading to a reduced Th1 response (less production of interferon-γ), and zinc deficiency leading to reduced Th2 response (less production in interleukin-6). Interestingly, in vitamin A deficient infants, the circulating neopterin concentrations were elevated, indicating a higher interferon-g production in vivo.

However, supplementing infants for 6 months with zinc, iron or β-carotene also led to different immune responses. For example, supplementation with only iron for 6 months gave a strong Th1 stimulus, with a significant higher production of interferon-γ. In contrast, interleukin-6 (a Th2 type cytokine) was lower in the infants receiving iron. Addition of zinc to the iron supplement reduced the effect on the immune modulation, with lower production of interferon-γ and higher production of interleukin-6 (although not yet reaching the levels of the placebo group).



But perhaps the most striking and important interaction on functional level we found when supplementing pregnant women with zinc or β-carotene in addition to standard iron and folic acid. We assessed the number of episodes with diarrhea and fever during the first 6 months after birth, as well as the immune reactivity of the newborns at 6 months after birth, and found that the addition of zinc or β-carotene to the iron-folic acid supplements for the mothers had profound effect of the newborn's immune system[3]. Children born from mothers supplemented with zinc had a significant higher interleukin-6 production, whereas children born from mothers supplemented with β-carotene had lower interferon-γ production.

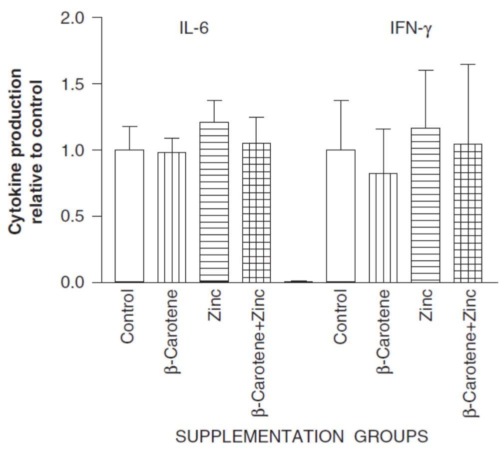
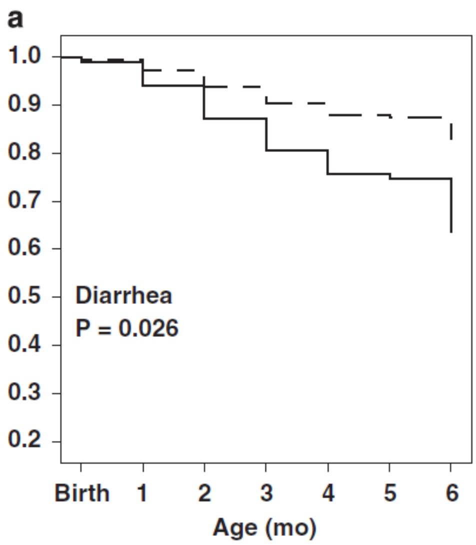


Figure 3 *Ex vivo* production of IL-6 and IFN-γ in infants at 6 months of age, born from mothers supplemented during pregnancy with β-carotene and/or zinc, in addition to iron and folic acid, relative to control group. Bars represent 95% confidence interval.

Both effects point towards a shift in the immune responses to favor Th2 responses[4]. As all women received iron and folic acid during pregnancy, we cannot answer the question on whether iron given during pregnancy will lead towards a shift in immune responses favoring a Th1 response (as seen in the infant study), and that this shift is restored by zinc or β-carotene, or that the effect of zinc and β-carotene is independent of the supplemented iron. Interestingly, a stronger Th2 response in infancy has been linked to atopy and allergies, and hence, addition of zinc or b-carotene to standard iron and folic acid supplement for pregnant women might have an increase in allergy prevalence as offset[4]. But given the millions of pregnant women worldwide taking vitamin and mineral supplements, more research into this area is highly warranted.

But from a public health point of view, the most important finding of the study was that children who were born from mothers receiving zinc had a significant lower risk for getting diarrhea during the first 6 months of life. Diarrhea is still one of the main causes of death in young children, and addition of zinc to the standard iron and folic acid supplements for pregnant women could be one option to reduce the prevalence of diarrhea in children. It is likely that the effects of prenatal supplementation last much longer than 3 – 6 months after birth, and may even have consequences for diseases in adult life. Therefore, there is an urgent need to better understand the effect of prenatal micronutrient status, and effects of supplementation during pregnancy and early childhood on neonatal immune responses and what this means for long-term survival and disease susceptibility.



6.2.3. Micronutrients and the acute phase response.

One problem with the immune response, or more specifically with the acute phase response, is that many protein concentrations are altered. The acute phase response is a generalized reaction of the body to inflammation. Proteins whose concentrations change as part of the acute phase response are referred to as acute phase proteins. Two commonly used acute phase proteins measured are C-reactive protein (CRP) and alpha-1 acid glycoprotein (AGP). CRP concentrations rise rapidly after the onset of the inflammation, but returns to normal levels within a few weeks. In contrast AGP concentrations begin to increase only later after the onset of inflammation, but remain elevated well into convalescence; thus AGP can be elevated for weeks after the onset of the inflammation. But indicators of micronutrient status also change due to the acute phase protein. For example, ferritin concentrations are increased, and zinc and retinol concentrations are decreased during the acute phase response. It has been suggested that this response is a natural defense of the body against pathogens, so-called ‘nutritional immunity’[68]. But this also means that the determination of ‘micronutrient status’ is hampered. To see whether we could control for the acute phase response, and come to a better estimate of micronutrient status, we conducted several analyses. In the first, we showed that the ‘estimate’ for deficiency is affected by the acute phase response.

TABLE 3

Prevalence of micronutrient deficiencies for all infants, the infants with neither CRP or AGP raised, and with raised CRP only, raised AGP only or both CRP and AGP raised

		Groups according to raised CRP and/or AGP[1]			
Micronutrient deficiency[2]	Whole population	Neither CRP nor AGP raised	CRP raised	AGP raised	Both CRP and AGP raised
Anemia, %	50.2	49.7	49.2	53.5	51.4
(*n*)	(418)	(308)	(61)	(86)	(37)
Iron deficiency anemia, %	21.5	26.0	3.3**[3]	10.5*	2.7*
(*n*)	(418)	(308)	(61)	(86)	(37)
Vitamin A deficiency, %	58.2	53.6	84.4**	69.4#	84.2*
(*n*)	(256)	(194)	(32)	(49)	(19)
Insufficient vitamin A liver stores, %	72.3	74.7	66.7	61.7†	58.8
(*n*)	(238)	(178)	(30)	(47)	(17)
Zinc deficiency, %	12.9	11.0	33.3**	15.1	27.0*
(*n*)	(418)	(308)	(61)	(86)	(37)

Iron deficiency is under-estimated (because ferritin concentrations are increased during inflammation) and zinc and vitamin A deficiency prevalence are over-estimated because of inflammation. The magnitude of the under- or over-estimation depends on the prevalence of inflammation in a population, and the phase of the acute phase response. For example, in the late convalescence phase, with only AGP elevated, the effect on the biomarkers for micronutrient status is lower when both CRP and AGP are elevated.

In a later study, we tried to calculate correction factors for ferritin concentrations. By determining the state of inflammation, one could in principle correct the ‘elevated’ ferritin concentrations by a correction factor, to come to a concentration similar to a ‘non-inflammation’ state. For this, we obtained data from 32 different studies in infants, children, men and women. We estimated the increase in ferritin associated with inflammation using again CRP and AGP concentrations. We had data on a total of 8796 subjects. In the paper, we propose correction factors of 0.77, 0.53, and 0.75 for ferritin concentrations in the 3 different phases of inflammation. This means that ferritin concentrations almost double at the height of inflammation[69]. Currently, we are working on correction factors for plasma zinc concentrations and soluble transferrin receptor concentrations, as no correction factors have been proposed yet for these indicators.

6.4. Micronutrient interventions and cognitive development

Nutrition is one of many key factors affecting mental development of children. Despite a general decrease in the global prevalence, stunting still affects one third of the children under 5 years in the developing world, and several studies have documented associations between cognitive performance and stunting in young children[19, 70]. Several micronutrients have been implicated in cognitive development of children too. Deficiencies of these micronutrients such as iron, folate or iodine can lead to impaired cognitive functions, with changes sometimes being permanent[71, 72]. Iron deficiency anemia for example has been associated with poorer cognitive performance and some recent systematic reviews showed evidence for a positive effect of iron supplementation on different measures of cognition in anemic and non-anemic children, on attention and concentration in adolescents and women and beneficial effects of micronutrient interventions (food-based or supplementation) on short term memory[73-75].

In the FORISCA project (described above), we measured cognitive performance in school children receiving fortified rice. Children aged >5 years are often omitted from public health programs, as programs typically focus on early child development. Yet, some areas of the brain areas and higher cognitive functions continue to develop throughout childhood and adolescence [76]. The myelination of frontal lobes which are thought to be responsible for executive, “higher-order” cognitive activities, starts around 6 months of age, but continues until adulthood. Changes in the volumes of cortical gray and white matters that are significantly correlated to children’s performance on a verbal learning task, occur during childhood and adolescence.

Therefore, we were interested whether nutritional status, micronutrient status and consumption of fortified rice could affect cognition. We used 3 different tests to measure cognition. The first test was

the Raven’s Colored Progressive Matrices test, which uses 36 pictures with a pattern and children need to identify the missing piece out of a choice of six different options. The patterns increase in difficulty. Raven's Matrices measure two complementary components of general intelligence: the capacity to think clearly and make sense of complex data (educative ability) and the capacity to store and reproduce information (reproductive ability).

The second test used was the block design test. Block design is a measure of problem solving to

assess executive function short-term memory and attention span. The third test was the picture completion test, which asks children to identify an error in a drawing. The tests were done by the school children under the supervision of students from the Faculty of Psychology, who have been trained on the test methods.

At baseline, cognitive scores were associated with stunting and micronutrient status[61]. Children with severe stunting scored significantly lower than non-stunted children in all tests (p<0.001 for all),

with a reduction in the scores reaching up to -2 points in RCPM test (12% of mean score), or -1.7 points in picture completion test (22% of mean score) after adjustment on all variables. Children with moderate stunting scored also significantly lower on 2 tests (Picture completion and Block design tests, both p<0.001) or tended to score lower on the RCPM test (p=0.053) when compared with non-stunted children.

Analysis of the micronutrient status of the children at baseline showed that marginal body iron stores (body iron≤4mg/kg) were associated with significantly lower scores in RCPM and picture completion tests (both p<0.05). After adjustment on all variables, boys with iron-deficiency anemia (IDA) scored significantly lower than boys with normal iron status in RCPM test (-1.46; p<0.05). Score difference was not significant for girls with IDA. For the picture completion test, children with normal iron status tended to score higher than iron-deficient children with anemia (-0.81; p=0.067) or without anemia (-0.49; p=0.064). Vitamin A, iodine or zinc deficiency were not significantly associated with cognitive performance at baseline in the FORISCA study[61].

Of course, we were also interested whether the intervention with fortified rice could improve cognitive performance. Cognition data at baseline and endline were available for 1796 children, who had a mean age of 10 years (ranging from 6 years to 16 years). All cognitive scores improved over the 6 months intervention. On average, the scores increased by 7 (54%), 3 (20%) and 3 (38%) points for block design, RCPM, and picture completion tests respectively, an effect we attribute to familiarization with the tests by the children. But the intervention also had a significant overall impact on block design scores (P=0.003). Improvements in block design scores were significantly higher in children consuming UltraRice®original (β=1.17, P=0.03) compared to the 2 other types of fortified rice and placebo. No significant difference in RCPM scores or picture completion scores was found between the intervention groups.

It appears perhaps surprising that the study found only an effect of UltraRice®Original on cognitive performance, and not of the two other types of fortified rice. But UltraRice®Original contained the highest concentration of iron, and this might perhaps explain the difference with the other 2 types of fortified rice.

We also investigated confounders for an impact of the intervention. For block design scores, only stunting had an influence on the impact of the intervention (P=0.006 for overall interaction) while intestinal parasite infection, inflammation and low body iron did not. The increase in block design score over time was higher in non-stunted children receiving NutriRice® compared to stunted children receiving NutriRice® (difference of scores -0.69, P=0.05).

For RCPM scores, parasite infection had a negative effect on the intervention (β: -0.71; P=0.045 for overall interaction). Among children receiving UltraRice®Original, RCPM scores increased more in children without parasites than in children with parasites (difference of scores -0.08, P=0.010), whereas the interaction between inflammation and overall RCPM scores tended to be negative too (β: -0.98; P=0.06). Interestingly, this is in parallel with the observed effects of fortified rice on iron status of children participating in the FORISCA study, which were only significant in children without inflammation[77]. This suggests that the acute-phase-response may disturb the response to fortified foods and therefore the impact on functional outcomes such as of cognitive performance.

For picture completion scores, both stunting and low body iron had a strong effect on the impact of the intervention (P<0.001 and P=0.001 respectively). In children receiving UltraRice®New, those with low body iron increased less in their picture completion scores compared to children with body iron ≥ 4 mg/kg (difference of scores 0.51, P=0.001). Similarly the scores of stunted children increased less compared to non-stunted children receiving UltraRice®Original (difference of scores -0.51, P=0.015).

These confounding effects of intestinal parasite infestation, stunting and inflammation on the impact of consuming fortified rice highlight the difficulties in measuring functional outcomes in an effectiveness trial, that is, in a ‘real-world’ setting. However, given the low prevalence of iron deficiency in this population, and the fact that low iron status was a standard confounder of the impact of the intervention, we believe that improving iron status in school children with a marginal iron status can have a big impact on cognitive performance. Indeed, in the study in Vietnam with fortified biscuits, we showed that children who received the fortified biscuits had significantly better scores for the Raven Colored Matrices (RCM) and Block Design tests[58]. The effect on the RCM test was especially strong in children who were anemic at baseline. In Vietnam, anemia is much more associated with a poor iron status than in Cambodia.

7. Summary and conclusions

In this dossier, I have given an overview of the research I have been implicated in over the last 20 years. The research can be roughly grouped into 2 main topics: treatment and prevention of acute malnutrition (both severe as moderate malnutrition) and micronutrient deficiencies.

The research on acute malnutrition started rather fundamental, focusing on immune reactivity and pharmacokinetics in children with SAM, but later turned much more practical, focusing on developing cheap yet high-quality, acceptable food products for the treatment and prevention of malnutrition. This latter work has been both challenging and rewarding. At the moment, in Vietnam the product development stage has been more or less finalized, meaning that the Ministry of Health is now moving towards scale-up of the production (current production capacity 100 MT/year). In Cambodia, product development is still ongoing, and will not be finished before 2018. However, once product development has been finished, my role will also be finished, as development of business plans or social-marketing are outside my scope of work. The work on complementary foods, such as in Vietnam and in Cambodia, is far from finished though. Although we have shown that with the provision of a good complementary food, such as WinFood-lite, one can stop growth faltering during the first months, it remains puzzling why we cannot stop this growth faltering to recur 6 or 7 months later. Perhaps other factors, such as the gut flora, play a yet unknown role in this, and clearly more research into this is needed.

The research on micronutrients has taken several interesting turns of the last decades. First of all, there is a clear change from 'supplementation' studies towards 'food-based' approaches such as food-to-food fortification or real fortification of staple foods with vitamins and minerals. This shift also implies a change from more biochemical research into more practical research, although the fundamental aspects of the research were never completely omitted. For example, the effects of rice fortified with iron and other micronutrients on hookworm infection is a clear example on how fundamental research can be incorporated into effectiveness trials. Another shift has been in the type of outcomes of trials: from indicators of micronutrient status towards functional outcomes. As such, the SEAMTIZI trials were very educational in that although one can improve status of some selected micronutrients, we should question the benefits of this if there is no impact on functional outcomes such as growth, morbidity or cognitive functioning. With this, I come to changes in immune response after nutritional interventions. We have shown that simple interventions such as iron, zinc or vitamin A supplementation have profound effects on the immune response. As yet, it is unclear how these changes can be translated into benefits (or harm) to the individual. There is fascinating research ongoing on the effect of vitamin

A on immune responses, but as far as I know, very little research is being done on the immune modulation aspects of e.g. iron or zinc. Yet, millions of people, especially pregnant women and young children, receive iron or zinc in the form of tablets or syrups. Therefore, in my view, this type of research should have the highest priority.

Other topics for research which should receive more attention include:

- Interactions between micronutrients. I have shown in this dossier how important interactions between micronutrients can be in terms of biochemical as well as functional outcomes. Unfortunately, interactions between micronutrients are currently unappreciated.
- Impact of nutrition interventions on gut flora. The gut flora has been shown to be a very important determinant of health and growth. Nutritional interventions are likely to lead to changes in the gut flora, but little is known about this. Currently we are analyzing data from the FORISCA trial to see whether fortified rice had an impact on gut flora. But more research is warranted in this field
- Impact of nutrition interventions on cognitive development of young children. With ~50% of the infants in low-income countries being iron deficient, the impact of nutrition interventions on long-term cognition should receive more attention. But not only iron is important. Hence, research into the long-term effects of providing a food supplement to infants would be needed to show policymakers the benefits of investing in nutrition for a new generation.

I hope to be involved in some of these topics above in the near future, depending of course on time, funding opportunities and continuation of existing collaborations.

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