Vitamin A, Iron and Zinc Deficiency in Indonesia.

Micronutrient Interactions and Effects of Supplementation.

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STELLINGEN / PROPOSITIONS

1) Concurrent deficiency of different micronutrients is the norm rather than the exception (this thesis).

2) Zinc plays an important role in the conversion of β-carotene to retinol (this thesis).

3) The subject "Nutrition" should only be practised from within a scientific discipline. On its own, the subject will not only lack in theoretical depth, but also in context and relevance.

4) Wat voor gedichten geldt, zou ook moeten gelden voor proefschriften en wetenschappelijke publicaties in het algemeen: "Een gedicht is beter naarmate men de woorden ervan minder merkt." (J.C. Bloem, 1887 – 1966)

5) "As in medicine the severity of surface symptoms and the severity of underlying pathology are not always in close correlation, so in sociology the drama of public events and the magnitude of structural change are not always in precise accord". (Clifford Geertz. The Interpretation of Cultures).

6) Als partners gezamelijk wetenschappelijk onderzoek doen, dan geldt $1 + 1 > 2$

7) Het is belangrijk voor zeilers op zee om de opkomst- en ondergangstijden van de heldere planeten uit het hoofd te kennen, zodat ze niet verward kunnen worden met de navigatieverlichting van grote schepen.

8) If a global one child policy would be launched tomorrow, many of the world's problems would solve itself over the next three generations.

Stellingen behorend bij het proefschrift
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Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de Rector Magnificus
van Wageningen Universiteit,
Prof.dr.ir. L. Speelman,
in het openbaar te verdedigen
op maandag 18 juni 2001
des namiddags om twee uur in de aula.

Samenstelling van de openbare vergadering ter verdediging van zijn proefschrift door F.T. Wieringa

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Prof.dr. H.P. Sauerwein, Universiteit van Amsterdam.

Prof.dr. R.E. Black, Johns Hopkins University, Baltimore, USA

STELLINGEN / PROPOSITIONS

1) In infants, iron supplementation should not be given without measures to improve vitamin A status also (this thesis).

2) Supplementation of single micronutrients in a population with concurrent deficiencies of various micronutrients has adverse effects (this thesis).

3) The statement of Primo Levi that "Zinc ...is not an element which says much to the imagination, it is grey and its salts are colourless, it is not toxic, nor does it produce striking chromatic reactions; in short, it is a boring metal" does not take into account the fascinating biochemical role of zinc.
(Primo Levi. The Periodic Table).

4) Genetically modified foods are an inappropriate answer to world hunger. The solutions should be political rather than scientific, and aimed at the abolition of the perpetuated unequal distribution of resources and technology.

5) Writing a thesis is like building a sailing boat. The merits but especially the faults will emerge only after launching.

6) Buitensporig ingewikkelde statistische berekeningen verhogen misschien wel de significantie van de bevindingen, maar verzwakken tegelijkertijd de sterkte van de conclusies.

7) Wij zijn slaven geworden van het snelle leven, dat onze gewoontes verstart, onze huizen binnendringt, en ons dwingt "Fast Food" te eten: Onze verdediging moet aan tafel beginnen met "Slow Food".
(Slow Food Manifest)

8) The observation: "The more Indonesia changes, the more it remains the same", has never been more true than the last four years. (F)
(Daan Mulder. Inside Indonesian Society).

Stellingen behorend bij het proefschrift
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Dijkhuizen, M.A. and Wieringa, F.T.

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This thesis is dedicated to all students,
in Indonesia and elsewhere,
who have given their lives
while standing up for their ideals,
searching for truth and justice.

They were killed
merely for striving towards a better world
for all of us.

ABSTRACT

Vitamin A, Iron and Zinc Deficiency in Indonesia. Interactions and Effects of Supplementation.

Ph.D. thesis by Marjoleine A. Dijkhuizen and Frank T. Wieringa, Division of Human Nutrition and Epidemiology, Wageningen University, The Netherlands. 18 June 2001.

Deficiencies of various micronutrients are prevalent in many developing countries, especially in infants and women. The research described in this thesis investigates the extent of deficiency of vitamin A, iron and zinc in pregnant and lactating women, and in infants in Indonesia. Furthermore, the effects of supplementation with β-carotene, iron and zinc on micronutrient status, growth, pregnancy outcome and immune function, and interactions between micronutrients are reported.

The research described in this thesis comprises a cross-sectional survey in 197 lactating mothers and their infants; a supplementation trial in 607 infants, who were supplemented from 4 months of age onwards; and a supplementation trial with 229 pregnant women, who were supplemented from 10-20 weeks gestational age until delivery, and followed with their newborn infants for 6 months post-partum.

An important finding is that deficiency of vitamin A, iron, and zinc are prevalent in infants and mothers, and that these deficiencies are likely to occur concomitantly. Furthermore, micronutrient status of the mother is strongly related to that of her infant, and breastmilk is a key connecting factor between mother and infant for vitamin A status.

Supplementation of infants with iron and zinc was effective in reducing the prevalence of anemia, and deficiencies of iron and zinc. Although there was some inhibitory effect of zinc on iron absorption, combined supplementation of iron and zinc was more beneficial than supplementation with one nutrient alone.

Supplementation of zinc in addition to β-carotene during pregnancy was found to improve vitamin A status in mothers and their infants 6 months post-partum. This indicates that zinc plays an important role in the conversion of β-carotene. Intriguingly, the synergistic effect of zinc on β-carotene supplementation is not apparent in infants, perhaps because infants have less capacity to metabolise or store β-carotene. Importantly, iron supplementation was found to have an antagonistic effect on vitamin A status in infants, possibly due to a redistribution of retinol to the liver. Therefore, iron supplementation in infants should not be given without measures to improve vitamin A status.

Supplementation of pregnant women with β-carotene and zinc improved birth weight, but only in boys. However, growth performance of neither the supplemented infants nor of the infants born of mothers supplemented during pregnancy, was improved, indicating that additional factors are involved in the growth impairment of these infants.

Various micronutrients have profound, albeit different effects on immune function. Vitamin A deficiency in infants was associated with lower *ex vivo* type-1 cytokine production, but higher *in vivo* macrophage activity, whereas in zinc deficient infants reduced white blood cell numbers, as well as lower type-2 cytokine production were seen. Supplementation of infants with iron resulted in higher type-1, and lower type-2 cytokine production, whilst supplementation with β-carotene and zinc appeared to have opposite effects on immune function.

In conclusion the research described in this thesis shows that concomitant deficiencies of various micronutrients are very prevalent, and that supplementation with single micronutrients is not optimal. The expected effectiveness of supplementation with one micronutrient will not be achieved if the utilisation of the micronutrient is impaired by deficiency of another micronutrient. Health benefits of supplementation will also fall short of expectations as long as deficiencies of other micronutrients are not addressed. Therefore supplementation with more than one micronutrient is recommended both for infants and for pregnant women.

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Note: Indicated in brackets is the researcher primarily responsible for the content of the chapter and who is also first author on the published or submitted paper.

CHAPTER 1

Introduction.

BACKGROUND

Malnutrition is still one of the major health problems in developing countries, affecting billions of people. Malnutrition can be caused not only by insufficient quantity of food, but also by poor food quality. The quality of the diet relates to the balance of amino acids and protein content, and to micronutrient content. Qualitative inadequacy of diets leading to deficiency of various micronutrients is very widespread, although signs or symptoms are often lacking. Micronutrient deficiency is therefore also referred to as the "hidden hunger"(1).

"Micronutrients" is the collective term used to describe vitamins and trace elements, which are required in only small amounts by the body. Vitamins may be present in the diet as such, but some vitamins can also be derived from provitamins. For instance vitamin A can be present in the food as retinol, or formed from provitamin A carotenoids. Trace elements, such as iron, zinc and copper serve many metabolic functions, often as part of protein complexes such as haemoglobin and metallo-enzymes.

Severe micronutrient deficiency often gives distinct signs and symptoms, and can be directly life threatening, but is not very prevalent. 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 (2). Micronutrient requirements during pregnancy and lactation are increased, and diets in developing countries often do not meet these higher requirements. 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. For example, children with marginal vitamin A status have higher morbidity and mortality of infectious diseases (3-5). Many studies have shown that deficiency of various micronutrients during pregnancy is associated with unfavourable pregnancy outcomes such as maternal mortality, congenital abnormalities and low birth weight (6-8).

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, and riboflavin, also warrant attention, not in the least because of the interactions among many micronutrients, and their interwoven roles in metabolic processes (9). People are likely to be deficient with respect to more than one micronutrient concurrently, as the same causative factors can underlie the aetiology of deficiency for different micronutrients. A cereal-based diet,

rich in phytate and low in animal products is common in most developing countries, including Indonesia. Such a diet predisposes to insufficient absorption of both iron and zinc (10). Furthermore, the amount of retinol and the absorption of provitamin A carotenoids from such a diet will be low (11). 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 (12).

Vitamin A.

Vitamin A can be obtained from the diet either directly as retinol, or derived from provitamin A precursors. The term "vitamin A" specifically refers to the fat-soluble substance of all-trans retinol, but generically used also includes other oxidation states of retinol, retinal and retinoic acid, and fatty acid ester forms of retinol, such as retinyl palmitate. Together, these compounds provide the biological basis of all aspects of vitamin A activity in the body, and each compound fulfils a specific role. However, retinol has a pivotal role and can be converted to all other compounds. Hopkins first identified the dietary factor leading to vitamin A deficiency in 1912, McCollum and Davies subsequently isolated vitamin A in 1913, and by 1931 its structure was completely elucidated by Karrer et al. (13,14). In addition to the retinoids, of which vitamin A compounds are a subgroup, there is the group of carotenoids, some of which are precursors to vitamin A. These are often referred to as the provitamin A carotenoids. Carotenoids are brightly coloured, and most colours in living nature are based on carotenoid pigments. Vitamin A itself is found only in animal tissues. Hence, carotenoids are far more abundant in nature than vitamin A, and are the main source of vitamin A in diets in developing countries (15). However the availability from the diet and conversion of provitamin A carotenoids to vitamin A are highly variable and often less than generally assumed (11).

Vitamin A has an important role in many metabolic processes. The most well-known function is in the visual processes in the eye. 11-*cis* Retinaldehyde, a derivative of retinol, is an essential component of rhodopsin, the pigment of the retina especially important in dim light vision (9). Hence, one of the first signs of vitamin A deficiency is night blindness. However, vitamin A, mostly as retinoic acid, is involved in the functioning of many other cells and tissues, including epithelial cells and cells of the immune system, and important for foetal development (16). Retinoic acid and derivatives play an important role in the regulation of cell differentiation and proliferation, by binding to the nuclear retinoic acid receptor and retinoid X receptor, which are then activated. The activated receptors form dimeric complexes that bind to specific elements of target genes, and regulate gene expression on transcriptional level (17).

Humans cannot synthesise vitamin A and have to rely on adequate intake of vitamin A or provitamin A carotenoids in the diet. Animal products generally contain vitamin A, and good sources are dairy products and liver, especially of fish. Plant foods contain no vitamin A, but large amounts of carotenoids, some of them with provitamin A activity. β-Carotene is the most important provitamin A carotenoid because its chemical structure can provide two vitamin A molecules, and therefore contributes most efficiently to vitamin A nutrition. α-Carotene and β-cryptoxanthin

also contribute, but to a lesser extent because with their chemical structure, they can only provide half the amount of vitamin A of β-carotene. Yellow and orange fruits and vegetables seem to be the best sources of provitamin A carotenoids in the diet (18). Although the β-carotene content of dark green leafy vegetables is high, they are a less efficient source of vitamin A than fruits because of lower bioavailability. The many factors affecting bioavailability and conversion of provitamin A carotenoids have been described and categorised but have not been fully quantified yet (11).

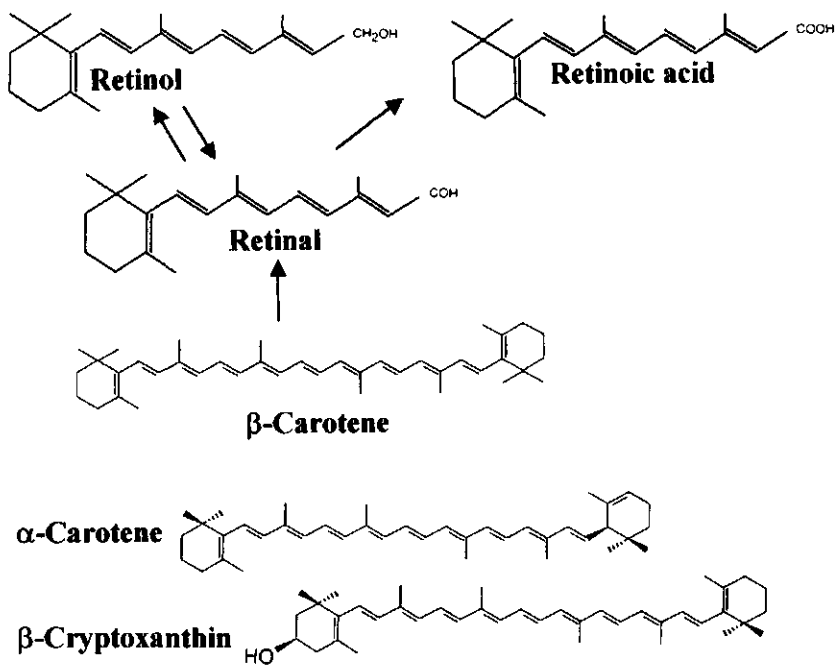
Retinol is readily absorbed (70%-90%) by the cells of the intestinal mucosa by facilitated diffusion. Carotenoids are often firmly encapsulated in insoluble complexes, and need to be released first before being available for absorption by the intestinal mucosal cells via a process of diffusion which does not seem to involve specific transporters. Hence absorption is less than for retinol and highly variable (5%-50%). In the mucosal cells, β-carotene can be converted to retinol by enzymatic cleavage, but in humans a significant proportion is absorbed intact into the body (16). As both retinol and carotenoids are fat-soluble, fat in the diet considerably improves absorption efficiency. Retinol and carotenoids are transported from the intestine to the tissues in chylomicrons, which are converted to chylomicron remnants in extra-hepatic tissues such as adipose tissue and muscle. In the liver, retinol is taken up by the parenchymal cells as retinyl esters, hydrolysed, and then bound to cellular retinol-binding protein (RBP). Retinol is then either secreted into the circulation bound to RBP and transthyrethin or transferred to the hepatic stellate cells where it is stored as retinyl esters, mostly as retinyl palmitate (19). Mobilisation of retinol from liver cells is still not completely understood, but involves close cooperation between the liver parenchymal and stellate cells, with the parenchymal cells producing most of the RBP necessary for mobilising retinol from hepatic stores. Adipose tissue appears to be an important storage site for β-carotene, and recently was shown also to store retinol (20).

Although vitamin A deficiency encompasses a wide range of changes in biochemistry, tissue and cell function and immunocompetence, the importance of vitamin A for public health has only recently been recognised (21). Historically, vitamin A deficiency was synonymous with a syndrome of ocular and epithelial damage, starting with night blindness and cumulating in destruction of the cornea (keratomalacia) and blindness. Already the Roman physician Celsus (25 BC-50 AD) used the term xerophthalmia to describe what we now know are the ocular signs of severe vitamin A deficiency (22). However, vitamin A deficiency is also important for immunity. This is illustrated by a meta-analysis of studies on vitamin A deficiency reporting 20%-30% reduction of mortality after vitamin A supplementation of children, even in populations without severe vitamin A deficiency (5).

Vitamin A status is determined by retinol stores in the liver. As these are difficult to measure directly, several other indicators are used to assess vitamin A status. Plasma or serum retinol concentration is the most widely used indicator. A plasma retinol concentration of <0.35 mol/L is considered deficient, and a plasma concentration of <0.70 mol/L is used to indicate marginal or subclinical vitamin A deficiency. In populations vitamin A deficiency is considered a severe public health problem if the prevalence of plasma retinol concentrations <0.70 µmol/L is >20%, and moderate if the prevalence is >10%. Other indicators of vitamin A status are plasma

concentrations of RBP, breast milk retinol concentrations, but stable isotope dilution techniques and several cytological and functional tests are also used to measure vitamin A status. These indicators are less often used because of technical difficulties or problems with validation. There are also some tests that offer an indirect measure of liver retinol stores. The relative dose response (RDR) measures the increase in plasma retinol concentration 5 hours after a small loading dose of retinol. The modified relative dose response (MRDR) test uses the ratio of the concentrations of 3,4-didehydroretinol to retinol, 5 hours after a loading dose of 3,4-didehydroretinol. When vitamin A liver stores are low, more retinol (RDR) or 3,4-didehydroretinol (MRDR) appears in the blood relative to initial or circulating plasma retinol concentrations (23).

Vitamin A supplementation is used worldwide to combat vitamin A deficiency. Intermittent high dose supplementation of children (100,000 IU between 6 and 12 months of age, 200,000 IU above 1 year of age) with capsules has been successful in many countries to virtually eliminate xerophthalmia. However, vitamin A can have toxic effects in high concentrations, and excessive dosing should be avoided. In pregnant women, vitamin A can have teratogenic effects on the foetus, so in women only low dosing (<10,000 IU/day or 25,000 IU/week) or a single high dose immediately post-partum (200,000 IU within 4 weeks of delivery) is recommended (24). β-Carotene has no toxic or teratogenic effects, but is not commonly used in supplementation.



Formulae of retinol, retinal, retinoic acid, and some carotenoids with pro-vitamin A activity.

Iron.

Iron is the one of the most abundant minerals on earth, and the human body requires only minute quantities. Yet iron deficiency is the most prevalent micronutrient deficiency, with about 2 billion people affected worldwide. Iron is easily oxidised, and in nature it is mainly found in the most oxidised state, Fe^{3+}. Iron is a transition metal; it can readily undergo reversible oxidation and reduction, and as such plays an important role in the metabolism of living organisms. Free iron is highly reactive, and living organisms have developed ingenious ways of storing and handling iron safely, primarily by binding iron to proteins. In adult humans, two-thirds of the iron in the body is present bound to haemoglobin in the erythrocytes. Haemoglobin is a protein essential for the transport of oxygen to the cells. Other important iron-binding proteins are myoglobin in muscle tissue, the cytochrome enzymes mainly in the mitochondria, and the proteins involved in iron transport and storage, transferrin and ferritin. Breast milk contains large amounts of the iron-binding protein lactoferrin (25).

Because of the poor solubility of Fe^{3+}, the availability of iron for living organisms is limited. Humans generally only absorb between 5% and 15% of the iron ingested. However, absorption of iron from the diet is highly variable and depends on many factors, including the iron status of the individual (26). One of the most important factors affecting iron absorption is the oxidation state of iron. Haem iron (Fe^{2+}) is more readily absorbed than non-haem iron (Fe^{3+}), but haem iron is only found in meat products. Interactions with other minerals such as calcium, zinc and copper can impair absorption by competitive uptake in the intestinal wall, whereas vitamin C and citrate can enhance uptake. Because iron is a cation, it can also form complexes in the gut lumen with other substances in food such as phytates, tannins, oxalates and fibres, making it inaccessible for absorption. Diets low in animal products, and comprising primarily cereals and vegetables (and thus also high in phytate, fibre and other chelating agents) are common in most developing countries. From such a diet only about 5% of the iron is absorbed, compared to over 15% from diets with abundant meat as consumed in most developed countries (27).

In cells, iron is bound to ferritin as a soluble complex, and macrophages, principally in the spleen, bone marrow and liver, are the largest depot of iron in the body after the erythrocytes. Mobilisation of iron from intracellular stores involves mainly transfer of iron from ferritin to transferrin, which transports iron in the plasma. The iron stores in the body contain enough iron to prevent iron deficiency for up to 3 years in adult men, but only up to 6 months in women, because of the iron losses through menstruation in women (27). The iron reserves of the newborn infant are usually depleted in 4 to 6 months, and the infant is then dependent on dietary intake.

Iron deficiency is defined by the absence of iron stores. One of the indicators of iron deficiency that is easiest to measure is haemoglobin concentration. Iron deficiency leads to reduced and iron deficient erythropoiesis, resulting in microcytic hypochromic anaemia. There are many other causes of anaemia however, and measuring only haemoglobin concentration does not differentiate between them. Also, haemoglobin concentrations decline only as an end stage of iron deficiency. Iron deficiency is most accurately determined by the absence of stainable iron in macrophages in bone marrow smears, however usually more practical indicators are used, which may give similar

information on iron status. In the absence of infection, plasma ferritin concentrations are directly related to iron stores. However, during infection the plasma concentrations of ferritin rise and are not directly related to iron status anymore (28). Also, the degree of iron deficiency cannot be assessed using plasma concentrations of ferritin, as these reach a nadir just before iron stores are completely empty. Another indicator that has been recently developed is serum soluble transferrin receptor concentration. It is considerably less affected by the inflammatory response. However, in replete and semi-replete situations, the concentration of serum soluble transferrin receptor seems to be less indicative of iron stores than ferritin. Also cut-off values are not clear yet. Many other indicators have been developed, but all have limitations because of the wide range of biochemical changes that occur during iron depletion. As none of the indicators is completely satisfactorily, the use of several indices of iron status simultaneously is probably necessary to obtain an accurate indication of iron status (29). In infancy, the situation is even more complex. In the first months of life, iron is relatively abundant due to neonatal iron stores, and iron is rapidly metabolised and recycled as the infant changes from foetal haemoglobin to normal haemoglobin. Also, iron requirements are high to support rapid growth. Plasma concentrations of ferritin are high at birth but decrease steadily during the first year of life, and haemoglobin concentrations in infancy are lower than in later life.

Iron deficiency leads to functional impairment of many tissues. Reduced erythropoiesis resulting in the development of anaemia is the most well-known example. However, iron deficiency of functional significance can already be present before erythropoiesis is affected. Iron deficiency impairs physical work capacity, not only as a result of reduced delivery of oxygen to the tissues because of anaemia, but also as a result of impaired cellular oxygen transport and oxidative metabolism in the tissues. The importance of iron for brain development is only recently emerging. Iron deficiency in infancy can lead to delayed psycho-motor development, and impaired learning. There is concern that these effects are, at least partially, irreversible (30). Also, iron plays an important role in the immune system, and iron deficiency leads to impaired immune function, especially a decreased cell-mediated immunological response (31).

Iron supplementation is commonly used to treat and prevent iron deficiency. It is standard practice in many countries to supplement pregnant women with iron (in combination with folic acid), because of the high prevalence of iron deficiency in pregnancy. Iron deficiency is associated with pregnancy complications, but a direct causal relationship has not been established (32). Iron supplementation programmes for children are being contemplated because of the high prevalence of iron deficiency and the important role of iron for brain development. Iron supplementation can cause gastro-intestinal irritation and obstipation, and ingestion of large amounts (>1 gram) has serious toxic effects and can be lethal in children.

Zinc.

Nutritional zinc deficiency was first described by Prasad et al. in relation to a distinct clinical syndrome of dwarfism and delayed sexual maturation in Iranian adolescent boys (33). Since then, the importance of zinc in human metabolism has been extensively documented. Zinc is an essential trace element involved in the

function of over 300 enzymes and proteins in the human body. These proteins are involved in a wide range of metabolic functions, including DNA transcription ("zinc-finger-motifs", a DNA binding domain with zinc as active metal, are present in many transcription factors), protein synthesis (zinc is part of tRNA synthetase, and has been detected in RNA, t-RNA, and ribosomes), catalytic enzymes such as alcohol dehydrogenase and alkaline phosphatase, hormone receptors (including the nuclear steroid and retinoid receptors), and in the stabilisation of cell membranes both zinc-containing protein complexes as well as membrane-bound zinc play a critical role(34). The biological role of zinc is always as a bivalent cation, and unlike iron, it does not undergo reduction or oxidation under physiological conditions, making zinc a stable component of protein complexes. Furthermore, zinc is a transition metal, allowing zinc to be bound in a large number of different constellations, enabling the formation of a wide variety of ligand binding sites. Therefore, zinc is a very versatile component of enzymes.

The absorption of zinc varies widely (5%-40%) and depends not only on the dietary content of zinc, but also on the bioavailability of zinc in the diet. Animal products are usually rich in readily available zinc, whereas zinc content in plant foods depends on soil zinc content. The bioavailability of zinc from plant foods can be markedly reduced by phytates, which form insoluble complexes with bivalent cations such as zinc, and other compounds which interfere with the absorption of zinc either by binding (folic acid) or competing for absorption (calcium or iron) (35). Zinc from galvanised cooking utensils and water pipes may be an important additional source of zinc (27).

In the body, zinc is subject to very strict homeostasis, and unlike iron or vitamin A, there are no known stores of zinc. However, zinc is present in all cells and tissues in the body, sometimes in high concentrations. In this context, the concept of type I and type II nutrients facilitates the understanding of zinc homeostasis and distribution. Type I nutrients are involved in certain specific metabolic functions, and deficiency will lead to specific clinical signs. There are well-defined stores, and in deficiency tissue concentrations will decrease. In contrast, type II nutrients are fundamental to the composition of cells and essential for the basic function of tissues. Hence, deficiency of a type II nutrient will primarily lead to generalised metabolic dysfunction, and eventually catabolism. Tissue concentrations of type II nutrients however are not decreased during deficiency. Furthermore, there are no well-defined stores of type II nutrients, and therefore requirements are continuous, but usually small. The primary response to deficiency is growth failure or loss of tissue. Examples of type II nutrients are essential amino acids, nitrogen, potassium, magnesium and also zinc (36).

Therefore, it is not surprising that the assessment of zinc status is wrought with difficulties. Plasma zinc concentration is often used as an indicator of zinc status, but does not satisfactorily reflect individual zinc status. The variation in plasma concentrations of zinc due to changes in zinc status are small, and can easily be overshadowed by other factors such as diurnal and inter-individual variation (37). However plasma zinc concentrations appear to give a reasonable indication of zinc status at the population level (38). Another problem is that plasma concentrations of

zinc decrease during infection, as a result of the acute phase response. Many other indicators of zinc status have been used, but all have severe limitations. In infants and children, improved growth performance after zinc supplementation remains the golden standard for demonstrating pre-existing zinc deficiency. Isotope dilution techniques can be used to measure body pools of zinc, which would indicate zinc status. However cost prevents widespread use of this technique.

Although the prevalence of zinc deficiency is difficult to assess, indirect indications suggest that zinc deficiency might be very widespread as the same dietary factors leading to iron deficiency also result in inadequate zinc nutriture. Even though the symptoms of zinc deficiency are not very specific, the consequences of zinc deficiency should not be underestimated. Zinc deficiency can lead to increased incidence and severity of infection because of impaired immunocompetence, impaired growth and development of children, increased incidence of pregnancy complications including increased maternal and perinatal mortality, and lower birth weight.

Supplementation with zinc is effective in alleviating or preventing zinc deficiency. However, zinc supplementation is not yet widely implemented, as the extent of zinc deficiency and the benefits of supplementation are not clear in many populations. Gastro-intestinal irritation can occur, especially at higher doses (>30 mg). Zinc is not very toxic, although doses of more than 60 mg/day in pregnant women caused premature births in one study (39). Rare instances of zinc poisoning (>1 g/day) are usually associated with food contaminated with zinc leached from galvanised food containers.

Micronutrients and growth.

Impaired growth is one of the most consistent signs of malnutrition, and is associated with poverty and poor health, and increased morbidity and mortality, and developmental impairment. In infants, 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. Growth can be measured in various ways, and expressed in different units. Height (or length) and weight are the most important measures, indicating linear and ponderal growth. They can be standardised against standard growth curves, and then expressed as Z-scores for age. Linear and ponderal growth are not necessarily simultaneous processes, in fact, there are indications that ponderal growth precedes linear growth (40). Also, growth impairment can exhibit specific patterns. This is best illustrated with the terms 'stunting' and 'wasting', with stunting being a length growth deficit, relative to the standard length for age ("short"), and 'wasting' an insufficient weight relative to height ("skinny"). However, a stunted child is not necessarily wasted and vice versa. The exact mechanisms behind growth faltering are not clear. 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 (41). The influence of nutrition on growth already starts before birth, and maternal nutritional status is an important factor, affecting birth weight, neonatal nutritional status and breast milk quality.

Zinc deficiency is classically associated with impaired growth, and many studies have found a positive effect of zinc supplementation on growth performance. A meta-analysis of such studies showed that zinc supplementation improved growth most in stunted children, children under the age of 24 months, and children with initial low plasma concentrations of zinc (42). Also, zinc supplementation has been shown to increase growth rates in severely malnourished children, but also in breast-fed infants from low-income families in France (43), and in growth limited, well-fed children in the United States and Canada (35).

The relation between vitamin A status and growth is less clear, although there is evidence that vitamin A deficiency is associated with stunting. Studies with vitamin A supplementation in children have had variable effects on growth, ranging from no effect at all, to significant increases in weight and/or height. A possible explanation for these differing results is the concomitant existence of deficiency of several micronutrients, together impairing optimal growth and muting potential effects of supplementation (41). Also, vitamin A supplementation often reduces morbidity, and this will indirectly lead to improved growth. There are some reports that β-carotene supplementation can improve growth performance, independent of improvements in vitamin A status.

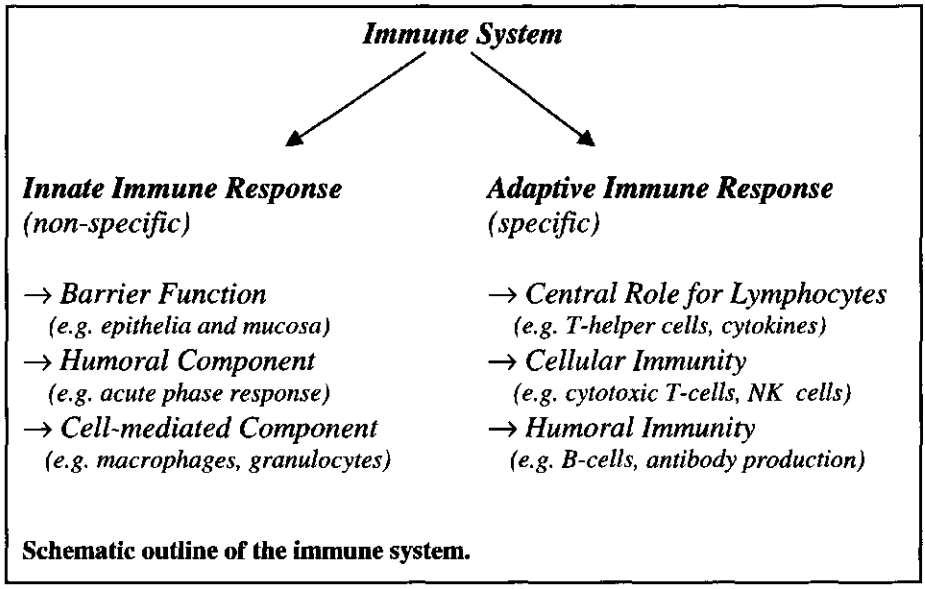
Although iron deficiency is associated with wasting, there is little evidence that iron supplementation can directly improve growth. Indirectly, the conditions that lead to iron deficiency such as hookworm infection also impair growth, and improved iron status can enhance appetite (41). There is some evidence that iron supplementation can improve growth in anaemic children (44).

Micronutrients and immune function.

The immune system consists of a non-specific or innate component, forming a constant defence mechanism that does not adapt to the invading agent, and a specific immune defence, capable of responding in an antigen-specific way. The most basic innate defence 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. Responses induced by cytokines can be very specific such as proliferation or activation of particular subpopulations of T-cells, or more generalised such as fever or suppression of erythropoiesis.



Schematic outline of the immune system.

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. Also, via breast milk, the infant acquires considerable amounts of immunoglobulins (mostly sIgA) and perhaps also other humoral and cellular components of the mother's immune defence. The protective effect of sIgA in breast milk is restricted to the gut, but the precise role of the other components remains unclear.

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 has been shown to increase morbidity and mortality of infectious diseases, and vitamin A supplementation in children can reduce mortality by as much as 20%-30% (45). 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 (46-48). Other studies have found overproduction of IFN-γ and reduced antibody production, fitting with a Th1 predominance in vitamin A

deficiency (49,50). In humans with vitamin A deficiency, decreased cell-mediated (type-1) immune function, reduced natural killer cell activity, impaired phagocytosis, reduced type-1 cytokine production and reduced antibody titres, have all been reported (51). Hence, the underlying immune defects in vitamin A deficiency remain elusive.

Severe zinc deficiency is accompanied by a marked increase in susceptibility to infections, but the effects of marginal zinc status are less well documented (52). In population studies, zinc supplementation gave a marked reduction in morbidity of diarrhoeal diseases, respiratory infections, as well as malaria (53-56). 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 cytokine production and antibody response (53,57). Zinc has been shown to affect the function of T-cells directly at the cellular level (58). Furthermore, animal studies have shown that prenatal zinc deficiency can result in a persistent disturbance of the offspring's immune system (52).

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 anaemia is present (59). 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. In humans, anaemia and iron deficiency are both associated with depressed cell-mediated immunity (59,60). Studies in animals suggest that an inadequate supply of iron restricts optimal T-lymphocyte proliferation. Humoral immune function or antibody response is probably less impaired. To complicate matters, supplementation with iron can enhance immune reactivity to such an extent that damage arises from the exacerbated inflammatory response (61). In population studies, the effects of iron supplementation on immune function has not been consistent, with some studies reporting increased immunocompetence while others report increased incidence and severity of infections (56,62).

Interactions between micronutrients.

Interactions among micronutrients occur at several different levels, but are often disregarded for the sake of simplicity, and because they are often difficult to study or quantify. Interactions may occur in food, in the absorption phase or once absorbed in the body. Food is a complex mixture of nutrients and other substances, and storage and cooking may cause losses of the more reactive micronutrients. In the absorption phase, food is digested, and nutrients are released to enable absorption. During digestion, micronutrients can affect the availability or absorption of other micronutrients, e.g. by enhancement of solubility, by competitive uptake, or by modulation of gut function. For example, iron bioavailability is enhanced by concurrent consumption of vitamin C, as vitamin C reduces Fe^{3+} to the more soluble Fe^{2+}. Bioavailability of both iron and zinc is reduced by high calcium intake, as they all compete for absorption via the same bivalent ion channel (63-65). In the same way, iron and zinc can mutually antagonise the absorption of each other, especially when present in higher concentrations (66). The integrity of intestinal mucosa can be impaired in vitamin A deficiency and zinc deficiency, thereby reducing nutrient uptake.

Once absorbed in the body, metabolic interactions can play an important role. If micronutrients function in the same metabolic pathway, deficiency of one micronutrient may impair the utilisation of the other micronutrient or micronutrients. For example, vitamin B_{12}, iron and folate are all needed in erythropoiesis, and deficiency of one of these micronutrients will lead to a dysfunction of the whole erythropoietic pathway, resulting in anaemia. In this manner one micronutrient deficiency can also mask the presence of deficiency of one or more other micronutrients, and correction of only one micronutrient deficiency will be ineffective. Another example is in the visual processes of the eye. Vitamin A is necessary for the synthesis of retinal, whilst zinc is part of the retinal dehydrogenase enzyme. Deficiency of either can thus give rise to night blindness (67). Another mechanism of interaction between vitamin A and zinc is via RBP. Zinc is essential for RBP synthesis in the liver, and RBP is essential for transport of vitamin A from the liver to peripheral tissues (68,69). Hence, severe zinc deficiency can lead to impaired vitamin A transport. Some metabolic interactions, such as the role of vitamin A in iron metabolism, have not yet been completely elucidated. Vitamin A can reduce the prevalence of anaemia, and improves the utilisation of iron (70,71). Also, zinc metallo-enzymes are involved in the conversion of β-carotene to retinol, suggesting another potential mechanism for interaction between vitamin A and zinc (9). However, the exact mechanisms are still unclear.

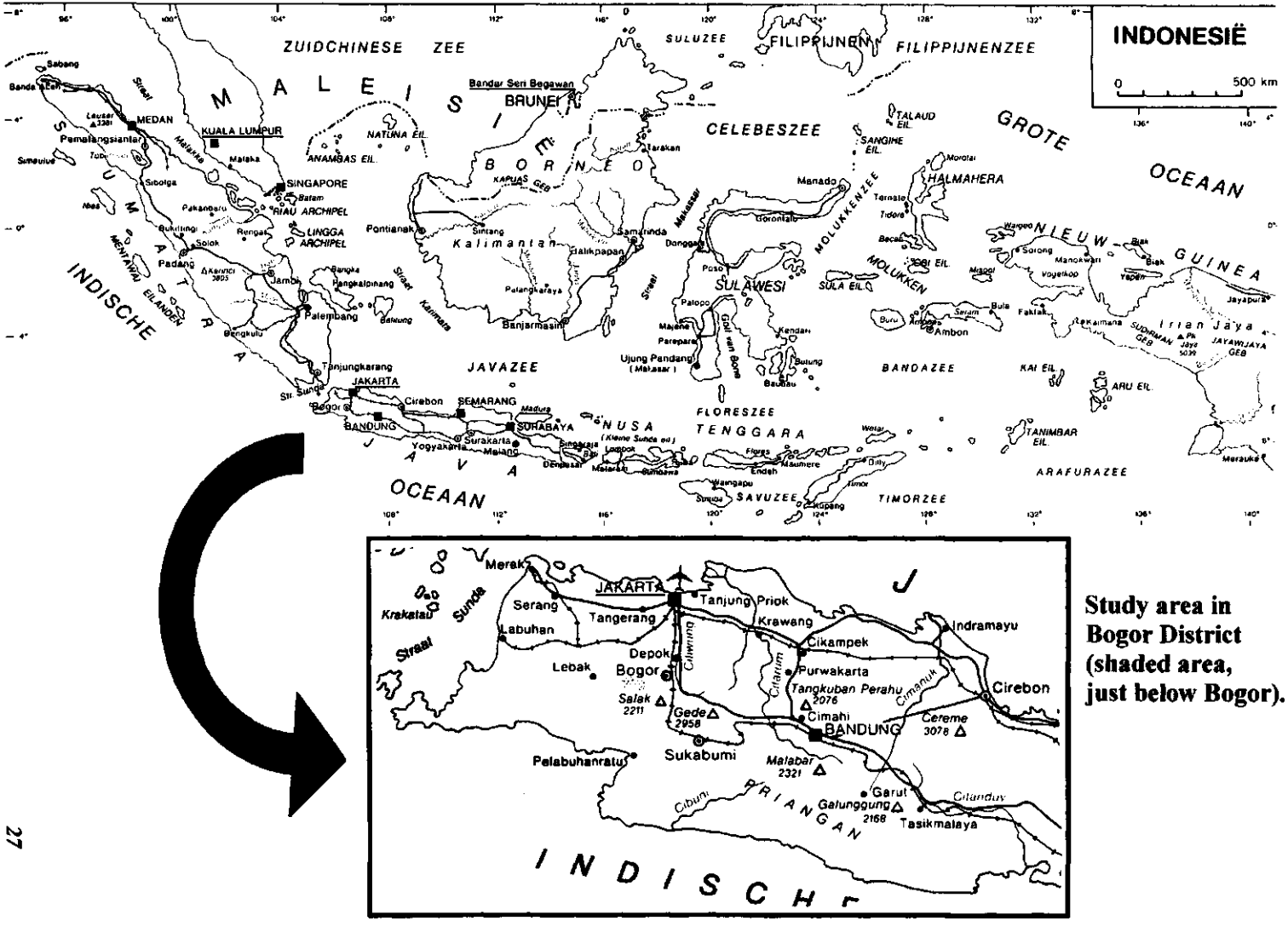
The effects of interactions between micronutrients can be exacerbated when micronutrients are supplemented in relatively high doses, and in situations where micronutrient nutrition is already marginal or deficient. For example, iron supplementation can impair zinc uptake, and this can induce zinc deficiency in populations with marginal zinc nutriture. Deficiency of several micronutrients often occur concomitantly in the same populations or even in the same person. This is not only because of the above mentioned interactions between micronutrients, but also because the same dietary factors leading to deficiency of one micronutrient often cause deficiency of other micronutrients. A rice-based diet, low in animal products as is common in Indonesia for instance, predisposes to both iron and zinc deficiency because of the high phytate content. Furthermore, the low fat content of the diet combined with the low intake of animal products can also cause inadequate vitamin A intake and absorption.

DESCRIPTION OF THE STUDY

Study site.

The studies described in this thesis were all conducted in kecematan (subdistrict) Cibungbulang of kabupaten (district) Bogor, West Java, Indonesia between September 1996 and December 1999. The area is rural, with most people dependent on farming for income and produce, and traditional in the sense that local traditions and customs are still important. Also, the traditional Sundanese diet consisting of mainly rice and vegetables, with some fish, tahu or tempe, and very little meat is adhered to. It is a fertile area, with three rice crops a year. The population is almost entirely Muslim. The study area fell under the responsibility of two health centres (Puskesmas). For the cross-sectional study, lactating mothers and their infants were recruited from two adjacent villages (Situ Udik and Situ Ilir). For the intervention study in infants, an additional four villages were included to supply a sufficient number of subjects (Sukamaju, Cemplang, Galuga and Dukuh). All these six villages fell under the responsibility of the same Puskesmas. For the intervention trial with pregnant women, another seven villages (Ciaruteun Udik, Leuweungkolot, Cimanggu I and II, Cibatok I and II, and Girimulya) were included. These were adjacent to the first six villages, but fell under the responsibility of the other Puskesmas.

In the course of this project, the political and economical situation in Indonesia changed dramatically. In the summer of 1997, an economic crisis was developing in the whole South-East Asian region, but at the start of the intervention trial in infants, the consequences were not yet apparent in the villages. At the beginning of 1998, the economic situation deteriorated quickly, and this economic crisis was felt intensely throughout Indonesia. By this time, the Indonesian rupiah had fallen from an exchange rate of 1,200 to 1 Dfl. to less than 7,000 to 1 Dfl. Imports stagnated, and millions of workers were made redundant. Several banks went bankrupt, and many shops and workplaces were simply closed. For instance in Cilebut, a small village of artisans and craftsmen and a regional centre for boatbuilding near Bogor, nearly 50% of the men became unemployed within two months (personal observations). In May 1998, after 32 years of being in power, President Suharto stepped down after a week of fierce riots, following the shooting on the Trisakti-university campus of 6 students, of whom 4 died. The Vice-President, Mr. Habibie, was appointed President, and new elections were called for. The year that followed was dominated by political changes, commonly referred to as "reformasi", but also the continuing economic crisis. After the elections of spring 1999, with a new President and Vice-President, Indonesians felt ready to make a new start. However, at the writing of this thesis (spring 2001), the economical problems have not yet been resolved, and the new political era has not yet brought the changes that were expected of it (72).



Study area in Bogor District (shaded area, just below Bogor).

OUTLINE OF THE THESIS

Below, a brief outline of the thesis is given. The studies described in this thesis were conducted between November 1996 and January 2000. For each chapter the subject of the research described in the paper is specified.

Chapter 2 describes the results of the cross-sectional survey with regard to micronutrient status of lactating mothers and their infants.

Chapter 3 describes immune response of Indonesian infants in relation to micronutrient status.

Chapter 4 describes the effects of iron and zinc supplementation of Indonesian infants on micronutrient status and growth.

Chapter 5 reports the effect of iron supplementation on vitamin A status in Indonesian infants.

Chapter 6 describes immune function in Indonesian infants supplemented with β-carotene, iron and zinc.

Chapter 7 shows the effects of inflammation on indicators of micronutrient status in Indonesian infants.

Chapter 8 describes the effects of supplementation of Indonesian pregnant women with β-carotene and zinc, in combination with iron and folic acid on pregnancy outcome, including pregnancy complications and birth weight.

Chapter 9 reports the effects of supplementation during pregnancy with β-carotene and zinc, in combination with iron and folic acid on micronutrient status of mother and infant, and growth of the newborn 6 months post-partum.

Chapter 10 discusses the main findings of the studies described in this thesis, and draws conclusions with implications for policy, programmes, and future research.

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Djenkol.

During the cross-sectional survey described in this thesis, we were alarmed by the high prevalence of, what we thought were urinary tract infections, as indicated by bright red discolouration of urine samples of the mothers. However, dipstick testing of the urine samples showed neither blood, nor other signs of urinary tract infection. The only explanation we could think of at the time was that the urine was discoloured by food additives excreted via the urine. However, Professor R. Luyken drew our attention to a whole body of literature from the 1930's and 1940's on the subject of "djengkol". Djenkol beans (Pithecolobium lobatum) are edible beans, in taste and smell not unlike peteh beans, eaten in relatively large quantities by the Sundanese. A disadvantage of the bean however is the presence of a sulphur-containing amino acid, djenkolic acid, which not only gives rise to the peculiar smell of the beans, but also colours the urine red, and can give toxic cystitis and nephritis when too many djenkol beans have been eaten. Normally, "djenkol-poisoning" is rare and appears to be mild, although fatal cases in children have been reported. Small crystals of djenkolic acid can be found in the urinary tract of people with djenkol poisoning. Individual susceptibility to djenkol-poisoning varies widely, and most people know their own limit of how many beans can be eaten before side effects occur. Before consumption, the beans are usually fermented for a couple of days and then either roasted or braised with spices. Only one other bean is known that also contains djenkolic acid, the boea kabau (Pithecolobium bubalinum), which is used in cooking in Sumatra. Besides containing this toxic substance, djenkol beans are a good source of vitamin B_1 and make a rather tasty side dish of semur.

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CHAPTER 2

Concurrent Micronutrient Deficiencies in Lactating Mothers and Their Infants in Indonesia.

Marjoleine A. Dijkhuizen, Frank T. Wieringa, Clive E. West, Muherdiyantiningsih, Muhilal.

Am. J. Clin. Nutr. 2001; 73: 786-91.

ABSTRACT

Background: Deficiencies of vitamin A, iron and zinc are prevalent worldwide, affecting vulnerable groups such as lactating women and infants. However, the existence of concurrent deficiencies has received little attention.

Objective: The aim was to investigate the extent of coexistence of deficiencies of vitamin A, iron and zinc, and also the nutritional relationship between lactating mothers and their infants.

Design: In a cross-sectional survey in rural West Java, Indonesia, 155 lactating mothers and their healthy infants were assessed anthropometrically, and blood, urine and breast milk samples were obtained.

Results: Marginal vitamin A deficiency was found in 54% of the infants and 18% of the mothers. More than 50% of the mothers and infants were anemic, and 17% of the infants and 25% of the mothers were zinc deficient. There was a strong interrelation between the micronutrient status of mothers and infants, and the concentrations of retinol and β-carotene in breast milk. Vitamin A deficiency in infants led to an increased risk for anemia and zinc deficiency (odds ratios: 2.5 and 2.9, respectively), whereas in mothers the risk for anemia and iron deficiency (odds ratios: 3.8 and 4.8, respectively) increased. In infants, concentrations of insulin-like growth factor 1 were related to concentrations of plasma retinol and β-carotene but not to zinc.

Conclusions: Micronutrient deficiencies were prevalent in West Java. The micronutrient status of lactating mothers and that of their infants were closely related; breastmilk was a key connecting factor for vitamin A status. Furthermore, micronutrient deficiencies appeared to be the norm.

INTRODUCTION

Micronutrient deficiencies are still a major public health problem in many developing countries, with infants and pregnant women especially at risk (1). Infants warrant extra concern, because they require extra micronutrients to maintain optimal growth and development. In this context, the nutritional relation between lactating mothers and their infants is of special interest.

Deficiencies of vitamin A, iron and zinc often coexist and have independent and interacting effects on health, growth and immunocompetence (2,3). It is well known that severe vitamin A deficiency leads to xerophthalmia. However, vitamin A is important in many other tissues and metabolic processes, and the considerable effects of vitamin A deficiency on morbidity and mortality have become clear (4,5). It is important to note that in populations with only marginal vitamin A deficiency, effects on metabolism and immune function are already present (4). Also, vitamin A deficiency was shown to contribute to the development of anemia and stunting (6,7). People in developing countries derive most of their vitamin A from provitamin A carotenoids, of which β-carotene is the most important. However, absorption, conversion, and mobilization of carotenoids and retinol are variable and dependent on many factors (8).

Iron deficiency is the most important cause of nutritional anemia, and is the most common micronutrient deficiency worldwide; it leads to impairment of health, growth, development and performance (9). Iron supplementation is currently the most important tool for combating iron deficiency. However, a high intake of iron, especially as a supplement, was shown to be an antagonist to zinc absorption (3,10).

The extent of zinc deficiency and its consequences are not yet clear. Zinc status is difficult to assess because plasma zinc concentrations do not sufficiently reflect individual zinc status because of strong homeostasis (11). In infants, improved growth performance after zinc supplementation is the most accurate measure of preexisting zinc deficiency but, on a population level, plasma zinc is still the most practical and reliable indicator of zinc status (12).

The manifestations of zinc deficiency range from an increased incidence and severity of infection and impaired growth and development of children to pregnancy complications, low birth weight and increased perinatal mortality (13-16). Zinc is also thought to play a role in vitamin A and β-carotene metabolism. Zinc supplements were shown previously to improve dark adaptation and intestinal integrity and zinc deficiency was found to aggravate the clinical effects of vitamin A deficiency (2).

Most public health and nutrition programs focus on one micronutrient only, whereas many populations can be expected to be deficient in several micronutrients at the same time. The aim of this study was to investigate the prevalence of concurrent micronutrient deficiencies in lactating mothers and their infants to elucidate the nutritional interrelation between the mothers and their infants, and the relation of micronutrient deficiencies with growth.

SUBJECTS AND METHODS

Subjects.

One hundred ninety-seven mothers-infants pairs (>90% of the eligible population of two rural villages in the Bogor District of West Java, Indonesia) were recruited over 2 mo (November and December 1996) by health volunteers to participate in the cross-sectional study. Forty-two of these pairs were excluded because of chronic or severe illness (n=2), severe clinical malnutrition (n=2), congenital anomalies (n=2), fever or other signs of mild systemic acute illness (n=15), twin birth (n=1), or incomplete data (20x). Complete data sets from 155 mother-infant pairs were available for statistical analyses. The excluded mother-infant pairs did not differ significantly from the subjects for whom data are reported. As is customary in Indonesia, all mothers were breast-feeding their infants, but most not exclusively so.

Mothers were informed of the procedures and purpose of the study. After written informed consent was given by the mother, infants and mothers were anthropometrically assessed, and a short history concerning socioeconomic status, dietary and lactation habits, and health was taken. Furthermore, a series of blood, urine, and breast milk samples from both the mothers and the infants were obtained as completely as possible. The protocol was approved by the Ethical Committee of the National Health Research and Development Institute of Indonesia and by the Ethical Committee of the Royal Netherlands Academy of Arts and Sciences.

Methods.

Anthropometry included measurement of weight, height and midupper arm circumference by a trained anthropometrist using standard methods. z scores for weight and height [weight-for-age (WAZ), height-for-age (HAZ) and weight-for-height (WHZ)] were calculated by using EPI-Info (version 6.02; Centers for Disease Control and Prevention, Atlanta) with the use of the WHO recommended growth curves (17). A fasting 3-mL venous blood sample was taken from the mothers and a non-fasting 5-mL venous blood sample was taken from the infants. A closed-tube heparin-containing vacuum system was used to avoid zinc contamination. Blood samples were stored immediately at 4°C to prevent microhemolysis and were separated within 5 h.

Breast milk was obtained from the right breast, 45-60 min after the last feeding from that breast. The breast was completely expressed and all milk collected. The manual breast milk pumps and containers were washed with acid to prevent zinc contamination (18). Urine samples were collected in acid-washed containers (mothers: midstream samples; infants: samples taken after the genital area was washed).

Zinc in blood, urine, and breastmilk was analyzed with flame atomic absorption spectrophotometry (Varian Australia Ltd, Clayton South, Australia), by using certified control sera (J. Versieck, Department of Internal Medicine, University Hospital, Gent, Belgium) as a quality control. For plasma and urinary zinc, the inter-assay CV was typically <5%, however breast milk zinc analyses showed more variability (10%), probably because of matrix effects. Urinary zinc concentrations were measured in casual urine samples in 104 infants and 130 mothers.

Retinol and β-carotene were analyzed using standard HPLC procedures. Hemoglobin concentrations were measured by using the standard cyanoblue method (Humalyzer, Tanusstein, Germany). Hematocrit in blood and creamatocrit in breast milk – a measure of breast milk fat - were determined according to standard practice. The fat content of breast milk was calculated on the basis of the creamatocrit content according to the method of Lucas et al(19). Ferritin, C-reactive protein (CRP), and insulin-like growth factor-I (IGF-I) were measured using commercial enzyme-linked immunosorbent assay kits (MP-products, Amersfoort, The Netherlands). Urinary creatinine was measured colorimetrically (Randox, Antrim,UK) and retinol binding protein (RBP) turbidimetrically (Behring Diagnostics Benelux NV, Rijswijk, The Netherlands). Because a close interrelation between indicators of micronutrient status of mothers and their infants was expected, the relation between hemoglobin, and plasma retinol, β-carotene, and zinc concentrations in the mother-infant pairs was examined.

Because many commonly used indicators of micronutrient status [eg, plasma concentrations of zinc, retinol, ferritin and RBP (20)] are altered by the acute phase response, subjects were screened for the presence of inflammation (12,20). Because a clinical examination often does not exclude the presence of minor or low-grade chronic infections, subjects with an acute phase response - as indicated by CRP concentrations > 10 mg/L or ferritin concentrations > 150 μg/L (> 400 μg/L in infants aged <6-mo) – were excluded from the statistical analyses (21).

Statistical analysis.

Data were checked for normal distribution by using the Kolmogorov-Smirnov test of normality. Relationships were analyzed by using multiple linear regression analysis; confounders were controlled for when necessary by using a backward deletion procedure (threshold $P > 0.1$). When no confounders were found, Pearson's correlation were used. Odds ratios and CIs were determined by using chi-square tests. Differences between groups were checked using Student's *t* test for parametric or log-transformed variables. Statistical analysis was carried out with EPI-Info (version 6.02) and SPSS (version 7.5.2; SPSS Inc, Chicago) software packages.

RESULTS

The infants ranged in age from 2.4 to 10.5 mo (**Table 1**), with an equal age distribution for both sexes; the sample included 78 males and 77 females. The mean HAZ was negative (- 0.73) whereas the mean WHZ was positive (0.24), indicating that in general the infants were short but not wasted. Eleven infants (7%) had a HAZ < -2, whereas one infant (0.6%) had a WHZ < -2. An HAZ > 2 and WHZ > 2 were found in 1% and 3% of the infants, respectively. Furthermore, all z scores were significantly negatively correlated with age after sex was controlled for ($P < 0.01$; HAZ: $r = -0.34$, WHZ: $r = -0.29$, WAZ: $R = -0.48$), implying that growth faltering in these infants started within the first months of life and progresses with age, as observed frequently in developing countries.

TABLE 1
General characteristics of the study population.

	Mothers (n=155)	Infants (n=155)
Age[1]	25 y (15 - 41)	6.6 mo (2.4 - 10.5)
BMI (kg/m^2)[2]	21.2 (19.7 - 23.0)	—
z Scores		
Weight-for-age	—	- 0.36 ± 1.08
Height-for-age	—	- 0.73 ± 1.08
Weight-for-height	—	0.24 ± 0.88

[1] Median; range in parentheses.
[2] Median; interquartile range.
[3] x ± SD.

Micronutrient deficiencies were prevalent in both the infants and mothers studied; >50% of the infants and mothers being anemic, and >50% of the infants had marginal plasma retinol concentrations (<0.70 µmol/L) (**Table 2**). Seventeen infants and 12 mothers were excluded because of having an acute phase response: either elevated CRP concentrations only or both elevated CRP and ferritin concentrations; none had elevated ferritin concentrations only. As expected, plasma retinol concentrations were significantly lower, and plasma ferritin concentrations were significantly higher in the excluded subjects. Plasma zinc and RBP concentrations, however, did not differ significantly in the excluded subjects.

In the infants there was a strong, linear correlation between plasma retinol and RBP concentrations ($r = 0.71$, $P < 0.01$; multiple regression analysis, no confounders were found; **Figure 1**). There was a positive correlation between concentrations of plasma zinc and urinary zinc (related to creatinine) in infants only ($r = 0.23$, $P < 0.05$, Spearman's rank test). The urinary zinc concentration is considered a more sensitive indicator of early zinc deficiency than is the plasma zinc concentration (14); however, in the present study it was found to be less suitable in the field setting, where sampling conditions were difficult. Usually only casual urine samples can be obtained in a field setting and it is often difficult to obtain samples from young infants. These factors contribute to the large variation in urinary zinc concentrations; therefore, this measurement is not very useful as a marker for zinc status.

TABLE 2

Indicators of the micronutrient status of Indonesian infants and their lactating mothers and prevalence of micronutrient deficiencies[1].

	Infants		Mothers		Correlation between mothers and infants[2]
Plasma retinol concentration (μmol/L)[3]	0.68	± 0.21[4]	1.09	± 0.40	0.30 [3]
Proportion < 0.70 mol/L (%)	54		18		—
Plasma ß-carotene concentration (μmol/L)	0.04	(0.02 - 0.05)[5]	0.14	(0.09 - 0.21)	0.42 [3]
Plasma lutein concentration (μmol/L)	0.29	(0.20 - 0.44)	0.41	(0.30 - 0.57)	—
Plasma retinol binding protein concentration (μmol/L)[3]	0.71	(0.62 - 0.86)	—		—
Hemoglobin concentration (g/L)	106	± 12	117	± 13	0.28 [3]
Proportion anemic (%)[6]	57		52		—
Plasma ferritin concentration (μg/L)[3]	26.9	(10.2 - 56.3)	13.9	(6.1 - 30.4)	NS
Proportion iron deficient (%)[7]	20		29		—
Plasma zinc concentration (μmol/L)[3]	13.1	(11.5 - 15.0)	12.6	± 2.7	0.45 [3]
Proportion plasma zinc < 10.7 μmol/L (%)	17		25		—
Urinary zinc concentration (μmol/mg creatinine)	17.4	(12.3 - 26.4)	6.7	(3.8 - 10.9)	NS

[1] n = 155.

[2] Pearson's correlation coefficient (with linear regression analysis, no confounders were found), $P < 0.01$

[3] Subjects with an acute phase reaction (17 infants, 12 mothers and 27 mother-infant pairs) were excluded from statistical analysis.

[4] $\bar{x} \pm SD$

[5] Median; interquartile range in parentheses.

[6] Anemia was defined as hemoglobin concentration <110 g/L (in infants) and <120 g/L (mothers)

[7] Iron deficiency was defined as anemia and a plasma ferritin concentration < 15 μg/L

There was a strong correlation between concentrations of hemoglobin, plasma retinol, plasma β-carotene, and plasma zinc in the mother-infant pairs (**Table 2**). Similarly, in lactating mothers breast milk was an important link between the nutritional status indicators of mothers and infants. Breast milk concentrations of retinol and β-carotene were significantly correlated to those in plasma of both mothers and infants (**Table 3**), accounting to a large extent for the strong interrelation between the vitamin A status of the mothers and infants. Concentrations of zinc in breast milk, however, were not significantly correlated with those in the plasma of either the mothers or infants. The fat content of breast milk [calculated on the basis of a median creamatocrit content of 3.7%; (interquatile range 2.8-5.0%)], was significantly positively correlated with both breast milk retinol and β-carotene (r=0.53 and r=0.24 respectively; $P < 0.01$ by multiple regression analysis). However conflicting findings on changes in micronutrient concentration during lactation were reported previously (22-25). In the present study, there was no significant effect of the duration of lactation on retinol, β-carotene, or zinc concentrations in breast milk.

Vitamin A deficient infants had a 2.5-fold greater risk of anemia and a 2.9-fold greater risk of zinc deficiency than did non-deficient infants, whereas the vitamin A deficient mothers were especially at risk of anemia (3.8-fold greater risk) and iron deficiency (4.8-fold greater risk) (**Table 4**).

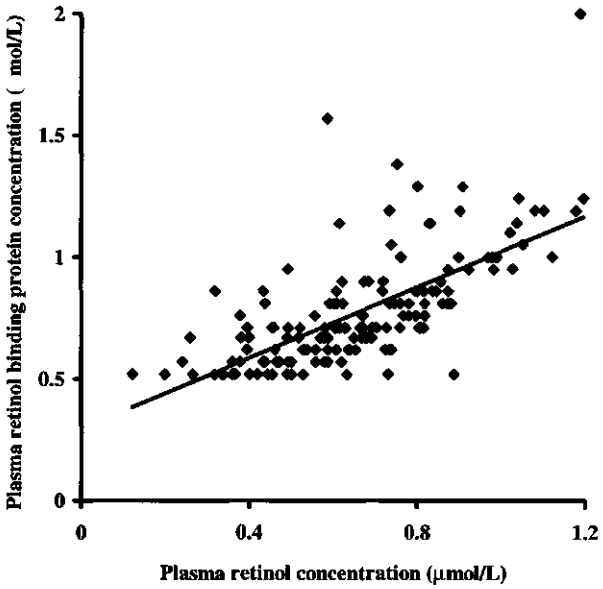

FIGURE 1. Relation between plasma concentrations of retinol and retinol binding protein in Indonesian infants. $y = 0.73x + 0.30$; $R^2 = 0.46$.

TABLE 3

Relationship between concentrations in breastmilk and plasma of retinol, ß-carotene, and zinc in Indonesian lactating mothers and their infants.

	Breast milk[1]	Correlation with plasma concentration	
		Mother[2]	Infant[3]
Fat (g/L)	21.3 (15.1 - 30.2)	—	—
Retinol concentration (µmol/L)	0.37 (0.21 - 0.67)	0.37 [4]	0.27 [4]
β-Carotene concentration (µmol/L)	0.006 (0.004 - 0.011)	0.49	0.58
Zinc concentration (µmol/L)	30.3 (20.5 - 47.2)	NS	NS

[1] Median; interquartile range in parenthesis. One hundred fifty breast milk samples were available for vitamin A analyses and 105 of these were analyzed for zinc.

[2] Partial correlation coefficient, with control for fat content, no other confounders were found. Subjects with missing creamatocrit values were excluded from analysis (12 mothers). $P < 0.01$

[3] Pearson's correlation coefficient (with linear regression analysis, no confounders were found). $P < 0.01$

[4] Subjects with an acute phase reaction were excluded from analysis (n-14 mothers, 17 infants).

TABLE 4

Risk of vitamin A deficient infants and mothers of having deficiencies of other micronutrients[1].

Micronutrient deficiency indicator	Vitamin A deficient infant OR (95% CI)	Vitamin A deficient mother OR (95% CI)
Anemia[2]	2.5 (1.3 - 5.0)	3.8 (1.4 - 10.0)
Iron deficiency[3]	2.4 (1.0 - 6.0)	4.8 (2.0 - 11.6)
Zinc deficiency[4]	2.9 (1.1 - 7.8)	1.9 (0.7 - 4.6)
Iron and/or zinc deficiency	2.6 (1.2 - 5.5)	2.8 (1.2 - 6.8)
Anemia and/or zinc deficiency	2.6 (1.3 - 5.3)	3.1 (1.1 - 8.9)

[1] Odds ratio; 95% CIs in parentheses. Subjects with an acute phase response were excluded from the statistical analysis.

[2] Defined as a hemoglobin concentration <110 g/L (in infants) and <120 g/L (in mothers).

[3] Defined as anemia and a plasma ferritin <15 µg/L

[4] Plasma zinc < 10.7 µmol/L

Long-term growth performance can be monitored by taking anthropometric measures, whereas IGF-1 concentrations provide a measure of current growth activity. In the infants studied, the median plasma IGF-1 concentration was 18.8 μg/L (interquartile range: 10.3 – 34.7) , and IGF-1 concentration were negatively correlated with age, as were WAZ, HAZ and WHZ scores (**Table 5**). Furthermore, a clear relation was observed between IGF-1 and plasma retinol and β-carotene concentrations, but not with zinc. No relationship was found between the indicators of micronutrient status and the anthropometric measurements.

TABLE 5

Relations between plasma concentrations of insulin-like growth factor 1 (IGF-1) in infants and indicators of anthropometric and micronutrient status[1]

	Correlation
Age	- 0.28 [2]
Z scores	
Weight-for-height	0.18 [3]
Weight-for-age	NS
Height-for-age	NS
Plasma retinol concentration	0.25 [4]
Plasma β-carotene concentration	0.21 [4]
Hemoglobin concentration	0.17 [3]
Plasma zinc concentration	NS

[1] n=155. The median IGF-1 concentration 18.8 μg/L (interquartile range 10.3 - 34.7).

[2] $P < 0.01$ (Pearson's correlation coefficient)

[3] $P < 0.05$ (partial correlation coefficients with control for age)

[4] $P < 0.01$ (partial correlation coefficients with control for age)

DISCUSSION

This study showed that deficiencies of vitamin A, iron and zinc occur concurrently in lactating mothers and their infants in rural villages in West Java, Indonesia. Vitamin A deficient mothers and infants had a 2–3-fold greater risk of also being deficient for iron or zinc than did non-deficient mothers and infants. Furthermore the interrelation between the micronutrient status of lactating mothers and their infants was shown clearly, with breast milk being a key connecting link, especially for vitamin A nutrition. One of the first effects of suboptimal nutrition in young infants is growth impairment, and the observed relation between plasma concentrations of IGF-1 and of retinol and β-carotene was intriguing.

Even though the population studied was not very deprived, merely rural and having traditional habits and diets, they have a high prevalence of marginal-to-severe micronutrient deficiencies, especially iron and vitamin A. On the basis of the current cutoff indicating zinc deficiency (plasma zinc <10.7 μmol/L; 26), the prevalence of zinc deficiency in the present study was almost similar to the prevalence of iron deficiency. This is despite the fact that zinc deficiency is less readily assessed than is iron deficiency. At the population level, the plasma zinc concentration is a useful indicator of zinc status (12). However current cutoff values for deficiency have been defined without considering the effect of infection on plasma zinc concentrations. Hence, the estimate of zinc deficiency in this population was conservative because subjects with an acute phase reaction were excluded.

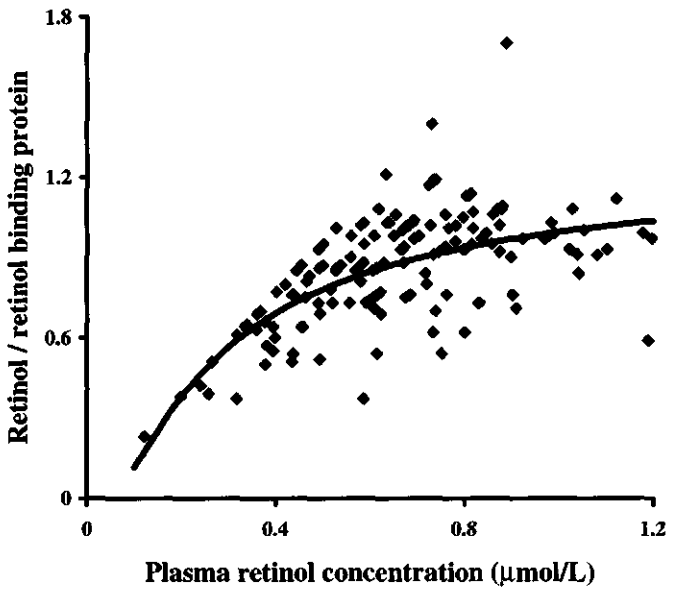

FIGURE 2. Relation between plasma retinol concentrations and the ratio of retinol to retinol binding protein in Indonesian infants. $\ln(y) = 0.24 - 0.24x^{-1}$; $R^2 = 0.54$.

The plasma RBP concentration was advocated recently as an alternative to the plasma retinol concentration for assessing vitamin A status. However, the relation between the plasma retinol concentration and the retinol-RBP ratio (**Figure 2**), showed that this ratio is constant and close to 1 only above marginal concentrations of retinol:

the ratio is <1 at lower plasma retinol concentrations. This lower ratio probably reflects an increase in plasma apo-RBP concentration in vitamin A deficiency (27). Hence, RBP becomes a less sensitive indicator for vitamin A status at lower plasma retinol concentrations. Therefore, substituting RBP for retinol as an indicator of vitamin A status may lead to an underestimate of deficiency. Methods that specifically measure holo-RBP circumvent this drawback (28), but are not yet generally available. It is important to establish reliable indicators for micronutrient status, especially when assessing a population for multiple micronutrient deficiencies, because confounding factors such as infection can easily complicate the overall picture.

Micronutrient deficiencies coexist and overlap because of common etiology and underlying mechanisms. For instance, a diet rich in phytate and low in animal proteins, as is common in most developing countries, including Indonesia, predisposes to insufficient intake and absorption of both iron and zinc (14, 29). Also direct interactions between micronutrients were described previously, such as the positive effect of vitamin A supplementation on iron-deficiency anemia (6), and the antagonistic effect of iron supplementation on zinc uptake (14). The mechanisms underlying these interactions are not yet fully understood. Focussing on several micronutrients instead of just one is important not only for treating micronutrient deficiencies but also for screening and identifying high-risk groups. This was illustrated clearly by the results reported here. Vitamin A deficient subjects had a much increased (2.4 - 4.8) risk of being deficient in iron also. The increased risk of a deficiency of both vitamin A and iron was described previously (30); thus hemoglobin screening is used to identify people at risk of vitamin A deficiency (6). Furthermore, this study showed an almost 3-fold greater risk of zinc deficiency in vitamin A deficient infants than in infants without a vitamin A deficiency, whereas there was a tendency toward an increased risk of zinc deficiency in vitamin A deficient mothers. Whether there is a common underlying cause of these micronutrient deficiencies, or whether one micronutrient deficiency leads to another deficiency could not be answered here.

The nutritional status of the mother is an important factor both prenatally and after birth. Prenatal maternal nutritional status affects birth weight, neonatal morbidity and mortality, and the micronutrient status of newborns. Postnatal maternal status can affect the quality of breast milk and thus the nutrient intake of infants (31). In the present study, indicators of the micronutrient status of the lactating mothers were clearly related to those of the infants. Concentrations of hemoglobin, plasma retinol, plasma β-carotene, and plasma zinc were all strongly correlated between the mother-infant pairs. For retinol and β-carotene this relationship was attributable to the breast milk link referred to above. The close relation between maternal vitamin A status and vitamin A concentration in breast milk was described previously (32, 33), but few data are available on the link between vitamin A concentration in breast milk and the vitamin A status of infants. The present study showed clearly that the concentrations of vitamin A and β-carotene in breast milk were closely related to those in plasma of the infants. No relation was found between zinc concentrations in the plasma of the mothers and in breast milk, reflecting earlier evidence for mammary zinc secretion is independent of maternal zinc status (23, 24).

Impaired growth in infants is in itself not hazardous to health but is associated with higher morbidity and mortality and with impaired cognitive and psychomotor development (34). The findings in the present study indicate that growth was suboptimal in these infants, with length more affected than weight. In accordance with findings in similar populations, HAZ scores were lower with increasing age. Growth failure is a consistent sign of zinc deficiency, and zinc supplementation was shown to improve growth, especially in stunted children (35). Ninh et al. (36) reported increased IGF-1 concentrations after zinc supplementation in stunted Vietnamese infants. In the present study, however, IGF-1 was found to be related to plasma concentrations of retinol and β-carotene in the infants, and to a lesser extent to hemoglobin concentrations, but not to plasma zinc concentrations.

There is clear evidence of a direct effect of vitamin A on cellular and tissue growth (37). However, supplementation with either retinol or β-carotene improved growth performance in some studies but not in others, and the underlying mechanisms are not clear (7,38-40). IGF-1 is a relatively new indicator for growth activity and the relationship between plasma concentrations of retinol and β-carotene and those of IGF-1 has not been reported. Thus, further investigation is required to establish whether this relation can be confirmed and to test whether the effect is causal. Other factors such as infection, nutrient status with respect to other nutrients, and genetic background could also be implicated (41). For instance, vitamin A status has profound effects on morbidity and could thereby indirectly influence growth (4).

The results of this study provide clear evidence that the concurrent occurence of deficiencies of a number of micronutrients is the norm rather than the exception. Of special significance is the strong interrelation between the micronutrient status of mothers and their infants; hower, the mechanisms involved have not yet been elucidated satisfactorily. In a future trial of β-carotene, iron and zinc supplementation, we will investigate interactions between vitamin A, iron and zinc and interrelations between micronutrient deficiencies in infants and pregnant women in West Java, Indonesia.

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Some Like It Hot .

There is an intriguing relation between climate and spiciness. Billman and Sherman analysed over 4,500 recipes, covering 36 countries for the use of spices in food. They found a strong correlation between the mean annual temperature of a country and the mean number of spices used, ranging from two in Norway, to ten in India. They argue that the greater spice use in warm climates is not because of the availability of spices, nor to disguise food spoilage, but that "the ultimate reason is most likely that spices help cleanse food of pathogens and thereby contribute to health, longevity and reproductive success of people who find their flavors enjoyable" Furthermore, they speculate that common spice combinations bring together complementary antimicrobials. There are several drawbacks to the study of Billing and Sherman however. For example, they only used cookbooks written in English, resulting in no recipes using indigenous African spices, Japanese wasabi, or Szechwan pepper that is often used in Chinese cooking. Also, being a cross-sectional survey of recipes, their "hygienic theory" can only be confirmed by infecting different traditional foods from around the world, ranging from say "Coq au Vin" to "Madras Chicken Curry", and following pathogen growth. However, that certain spices have antimicrobial as well as inflammatory modulating properties is without doubt. Curcumin for example inhibits the formation of prostaglandins, and eugenol, a spice principle from cloves, has anti-inflammatory properties, maybe explaining the custom of chewing on a clove to relieve tooth-ache.

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CHAPTER 3

Reduced Production of Pro-Inflammatory Cytokines in Vitamin A and Zinc Deficient Infants.

Frank T. Wieringa, Marjoleine A. Dijkhuizen, Johanna van der Ven-Jongekrijg, Clive E. West, Muhilal, Jos W.M. van der Meer.

Submitted for publication.

ABSTRACT

This study investigates the effects of vitamin A and zinc deficiency in infants on the production of pro-inflammatory cytokines. In 59 Indonesian infants, age 3 to 10 mo, the production of interferon-γ (IFN-γ), interleukin-12 (IL-12), interleukin-18 (IL-18) and interleukin-6 (IL-6) was measured after stimulation with lipopolysaccharide (LPS) and phytohaemagglutinin (PHA) in an *ex vivo* whole blood culture system. Circulating neopterin concentrations were determined as indicator of *in vivo* macrophage activity. Vitamin A deficient infants had significantly reduced *ex vivo* production of IFN-γ. Production of IFN-γ was strongly correlated to IL-12, but not to IL-18 . Intriguingly, vitamin A deficient infants had significantly raised circulating neopterin concentrations. Zinc deficiency was accompanied by significantly reduced white blood cell counts, as well as a significantly reduced *ex vivo* production of IL-6. Overall, this study shows that vitamin A deficiency and zinc deficiency have marked albeit divergent effects on the immunocompetence of infants.

INTRODUCTION

Micronutrient deficiencies are prevalent worldwide and especially affect vulnerable groups such as infants and pregnant women [1]. Syndromes as xerophthalmia in vitamin A deficiency and nutritional anemia are the most obvious manifestations of micronutrient deficiency. However, it is becoming increasingly clear that the effects of micronutrient deficiencies are more generalised and widespread. Profound effects on growth, development, and immunocompetence can be present in even marginal micronutrient deficiency [2,3]. For example, vitamin A-deficient populations have markedly increased rates of morbidity and mortality from infectious diseases, and vitamin A supplementation can reduce infant mortality by as much as 25%, even in populations with a low prevalence of xerophthalmia [2].

It is tempting to attribute the immunedefects at least in part to a disturbed response of pro-inflammatory cytokines. Studies in animals have given conflicting results on this issue. 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 [4-6]. Other studies have found overproduction of IFN-γ and reduced antibody production, fitting with a Th1 predominance in vitamin A deficiency [7,8].

In humans with vitamin A deficiency, decreased cell-mediated (type-1) immune function, reduced natural killer cell activity, impaired phagocytosis, reduced type-1 cytokine production and reduced antibody titres, have all been reported [9]. Hence, the underlying immune defects in vitamin A deficiency remain elusive.

Another micronutrient that has profound effects on the immune system is zinc. The same dietary factors leading to nutritional anemia, the most common micronutrient deficiency worldwide, contribute to inadequate zinc nurtiture, indicating that zinc deficiency may be very widespread also. In population studies, zinc supplementation gave a marked reduction in morbidity of diarrheal diseases, respiratory infections, as well as malaria [3,10,11]. Zinc deficiency in both humans and animals causes a striking depletion of both B- and T-cells, and also decreases numbers of neutrophilic granulocytes, natural killer cells and macrophages, as well as cytokine production and antibody response [3,12,13]. Zinc has also been shown to directly affect function of T-cells on the cellular level [14]. Furthermore, animal studies have shown that prenatal zinc deficiency can result in a persistent disturbance of the offspring's immune system.

This study investigates whether vitamin A deficiency and zinc deficiency in infants impair the ability to produce cytokines. The production of several pro-inflammatory cytokines was measured after stimulation of whole blood with LPS and PHA in an *ex vivo* culture system. Given the putative role of interferon-γ (IFN-γ), interleukin-12 (IL-12) and interleukin-18 (IL-18) in cell-mediated immunity, and interleukin-6 (IL-6) in humoral immunity, we investigated the production of these cytokines in a cohort of infants with carefully defined nutritional status. In addition, circulating neopterin concentrations were determined as indicator of *in vivo* macrophage activity induced by IFN-γ.

MATERIALS AND METHODS

Subjects and procedures.

Infants, 3 to 10 mo old, were recruited from two adjacent villages in rural West Java, Indonesia for a nutritional survey and further in depth immunological testing was done when field circumstances allowed (availability of electricity). All children studied were deemed healthy by clinical assessment. Mothers were informed of the procedures and purpose of the study. After written informed consent was given by the mother, a short history was obtained, infants were anthropometrically assessed, and a blood sample was taken from the infants.

Sample collection.

A non-fasting 5 mL venous blood sample was taken using a closed-tube vacuum system (Becton and Dickinson, Leiden, The Netherlands). The EDTA blood sample (2 mL) was used for hematological analyses as well as for the *ex vivo* whole blood cultures. The heparinized blood sample (3 mL) was used for the measurement of plasma concentrations of retinol, zinc, ferritin, C-reactive protein (CRP) and neopterin.

***Ex vivo* whole blood cytokine production.**

Cytokine production was measured using the *ex-vivo* whole blood cytokine production after stimulation assay as described by Van Crevel et al. [15]. The concentrations used, as well as the incubation time, were chosen to give an optimal response in this system. Within 15 min of collection of the blood sample, a mixture of phytohaemagglutinin (PHA-P, Sigma, Zwijndrecht, The Netherlands) in a final concentration of 30 mg/L, and lipopolysaccharide (LPS, *E. coli* serotype 055:b5; Sigma) in a final concentration of 10 μg/L, was added directly to the closed vacuum EDTA blood collection tube and then incubated at 37°C for 10 h. After incubation, supernatant was collected and cytokine concentrations determined. Blood was incubated with a combination of PHA and LPS in order to achieve a stimulation as complete as possible.

Hematological and biochemical analyses.

Hemoglobin concentration was measured by the standard cyanoblue method (Humalyzer, Germany). Total white blood cell (WBC) and differential counts were done by an experienced technician using standard microscopy methods. Retinol was analysed using high performance liquid chromatography (Thermo Separation Products, San Jose, CA, USA) and zinc was analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace-element free procedures, as described in an earlier paper [16]. The CV for both retinol and zinc analyses was <5%. Ferritin, CRP, and neopterin were measured using commercial ELISA-kits (MP Products, Amersfoort, The Netherlands) according to the guidelines of the manufacturer. The cytokines IFN-γ, IL-6, IL12-p70, and IL-18 were also measured with ELISA (Pelikine, CLB, Amsterdam, The Netherlands and (IL-18 only) R&D Systems, Minneapolis, MN, USA) according to the manufacturer's guidelines. IL-12 p70 was determined because this is the heterodimer responsible for the biological activity [17]. The CV for all ELISA assays was <10%.

Ethical approval.

The protocol was approved by the ethical committee of the National Health Research and Development Institute of Indonesia, and by the ethical committee of the Royal Netherlands Academy of Arts and Sciences.

Statistical Analysis.

Data was checked for normal distribution using Kolmogorov-Smirnov test of normality. For data not normally distributed, non-parametric tests were used. Relationships between not normally distributed data were analysed using Spearman's rank test. Differences between groups were analysed with the Student's t test for parametric data and Mann Whitney U test for non-parametric data. When necessary to control for confounding variables, analysis of covariance was used, with age as a covariate. Statistical analysis was carried out with SPSS 7.5.2 software package (SPSS Inc, Chicago, IL, USA).

RESULTS

Subjects, general characteristics and micronutrient status.

In total, blood for the measurement of *ex vivo* cytokine production was collected from 60 infants, and complete data was available from 59 infants. The infants (32 boys and 27 girls) ranged in age from 3 to 10 mo, and the age distribution was the same for both sexes (**Table 1**).

As the plasma concentrations of both retinol and zinc fall during an acute phase reaction, these indicators in the presence of inflammation, might not reliably reflect micronutrient status [18]. As clinical examination does often not exclude the presence of minor or low-grade chronic infection, seven infants with an acute phase response, as indicated by a plasma CRP concentration >10 mg/L, were excluded from the statistical analyses of micronutrient indicators.

Plasma retinol concentrations were significantly lower in the excluded subjects as expected (mean 0.44 vs 0.69 μmol/L, $P < 0.01$, Student's t-test), but plasma zinc and ferritin concentrations were not significantly different (Mann Whitney U).

Marginal deficiency of vitamin A was defined as a plasma retinol concentration <0.70 μmol/L. Zinc deficiency was defined as a plasma zinc concentration <9.95 μ mol/L (non-fasting blood samples) [19]. Of the 52 infants, 25 (48%) were vitamin A deficient, and 9 (17%) were zinc deficient (**Table 1**).

***Ex vivo* cytokine production reduced in vitamin A and zinc deficiency.**

The production of the pro-inflammatory cytokines IFN-γ, IL-12, IL-18, and IL-6 after *ex vivo* stimulation of whole blood with LPS and PHA was measured. There was a strong positive correlation between the production of IFN-γ, IL-12 and IL-6 (r's>0.47, $P < 0.001$, Spearman's rank). IL-18 production was negatively correlated to IL-12 (r= -0.42, $P < 0.01$) and IL-6 production (r=-0.30, $P < 0.05$, Spearman's rank). In vitamin A deficient infants the production of IFN-γ was significantly lower than in infants with adequate vitamin A status (**Fig.1**, $P < 0.05$, Mann Whitney U). Production of IL-12 was also lower in vitamin A deficient infants, but this effect failed to reach

Table 1. Indicators of micronutrient status in the infants, and characteristics of the vitamin A and zinc deficient groups.

	Infants	Vitamin A[3]		Zinc[3]	
		deficient	non-deficient	deficient	non-deficient
n	59	25	27	9	43
Plasma concentrations					
Retinol (μmol/L)[1]	0.66 (±0.24)	0.49 (±0.12)[4**]	0.88 (±0.14)[4**]	0.56 (±0.16)	0.72 (±0.24)
Zinc (μmol/L)[1]	12.6 (±2.8)	11.6 (±2.8)[4*]	13.3 (±2.5)[4*]	8.7 (±0.7)[4**]	13.3 (±2.3)[4**]
Ferritin (μg/L)[2]	20.7 (11.5 - 50.0)	19.2 (10.0 - 61.0)	17.9 (9.9 - 50.0)	14.7 (8.0 - 76.3)	19.8 (10.3 - 46.0)
Hemoglobin concentration (g/L)[1]	10.9 (±1.2)	10.6 (±1.3)	11.2 (±1.1)	10.7 (±1.1)	10.9 (±1.2)

[1] Mean (± SD).

[2] Median (IQR).

[3] Deficiencies were defined as plasma retinol concentration <0.70 μmol/L for (marginal) vitamin A deficiency and plasma zinc concentration <9.95 μmol/L for zinc deficiency. Infants (n=7) with plasma C-reactive protein concentrations >10 mg/L were excluded.

[4] Significant difference between groups,Student's t-test. ** P <0.01, * P <0.05

significance (**Fig.1**, $P < 0.10$, analysis of covariance, with age as covariate). There was a trend towards higher IL-12 production with increasing age ($P = 0.10$, Spearman's rank), whereas the production of the other cytokines was not related to age. In contrast to IL-12, there was no correlation of IL-18 production with IFN-γ production (Spearman's rank). Also, IL-18 production was not different between the vitamin A deficient and non-deficient groups (Mann Whitney U). IL-6 production in vitamin A deficient infants was also lower than in non-deficient infants, but not significantly (**Fig.1**, $P < 0.10$, Mann Whitney U).

Although only 9 infants were zinc deficient, IL-6 production was significantly decreased in the zinc deficient infants as compared to non-deficient infants (**Fig.2**, $P < 0.05$, Mann Whitney U). There was no significant effect of zinc deficiency on IFN-γ, IL-12, or IL-18 production, although there was a tendency towards lower production for all cytokines in zinc deficiency (**Fig.2**, Mann Whitney U). The exclusion of the 7 infants with a raised CRP did not change the observed relationships between cytokine production and vitamin A or zinc status.

White blood cell counts lower in zinc deficiency, but not in vitamin A deficiency.

WBC counts and differentiation were similar in the vitamin A deficient and non-deficient groups (**Table 2**). Zinc deficient infants however had significantly decreased WBC counts (**Table 2**, $P < 0.05$, Mann Whitney U), with especially lower counts of neutrophilic granulocytes ($P < 0.05$, Mann Whitney U). Eosinophilic granulocytes and lymphocyte counts also tended to be lower, but not significantly. Total lymphocyte, monocyte and neutrophilic granulocyte counts correlated significantly with IL-6 production ($r=0.37$, $P < 0.01$; $r=0.51$, $P < 0.01$; and $r=0.31$, $P < 0.05$ respectively, Spearman's rank), but not with the other cytokines. In a multiple regression model with the lower WBC differential counts included, zinc deficiency remained an important determinant of IL-6 production ($R= -0.23$, $P < 0.10$, multiple regression analysis).

Circulating neopterin higher in vitamin A deficiency.

Circulating plasma neopterin concentrations were significantly related to the age of the infants ($r=0.30$, $P < 0.05$, Spearman's rank). Vitamin A deficient infants had significantly higher neopterin concentrations than non-deficient infants (**Table 2**, $P < 0.05$, analysis of covariance, with age as covariate). There was no relation between zinc deficiency and circulating neopterin concentrations.

Iron status, as indicated by plasma ferritin concentration and hemoglobin, was neither correlated with any of the cytokines studied, nor with circulating neopterin concentrations.

DISCUSSION

In this study, infants with marginal vitamin A status had a reduced production of the type-1 cytokine IFN-γ in whole blood after stimulation with PHA and LPS. The production of IFN-γ is under the control of the more proximal cytokines such as IL-12 and IL-18. Of these, IL-12 showed a tendency of reduced production in vitamin A deficiency. However, the production of IL-18 in vitamin A deficient infants was not different from infants with a normal vitamin A status. These data, supported by the finding that IL-12 and IFN-γ production were strongly correlated, suggest that the fall in IFN-γ production in vitamin A deficiency is due to decreased production of IL-12, and not to IL-18 production since that appears not to be affected in vitamin A deficiency. The lower production of IFN-γ would imply less activation of the cell-mediated arm of the immune system.

In this respect, our finding that circulating neopterin concentrations were higher in the vitamin A deficient infants is intriguing. It is generally believed that elevated neopterin concentrations reflect increased macrophage activity, as it is regulated by IFN-γ [20]. How can this paradox of decreased *ex vivo* IFN-γ production and increased *in vivo* neopterin concentrations be explained? One explanation could be that the 10 h culture system used, reflects only the first non-proliferative phase of the cellular response. Whether more prolonged cultures with proliferating T-cells would give a different IFN-γ response remains to be investigated. However, in the field situation in which these studies were carried out, large blood samples, cell separation and prolonged incubation are not readily feasible. Another explanation for the apparent discrepancy of *ex vivo* IFN-γ production and circulation neopterin concentrations is that there is an upregulation of IFN-γ receptors and/or of the IFN-γ signalling pathway in vitamin A deficiency.

Irrespective of the mechanism, our findings suggest a type-1 cytokine dominance in a steady state, as indicated by elevation of neopterin concentrations, accompanied by an impairment of IFN-γ response during stimulation as may occur during infection. In this context, it is important to note that these infants were selected to have no acute phase reaction, to exclude coincident active infection or inflammation as much as possible, as this could modify both immune response and indicators of nutritional status.

Even while only 9 infants were zinc deficient, a significant reduction of IL-6 production in whole blood cultures after stimulation was found in this group. No significant effect of zinc deficiency on either IFN-γ, IL-12, or IL-18 production was found, but the small group size may have obscured an effect.

These results suggest a decreased type-2 cytokine response in zinc deficiency. Consistent with this finding are earlier reported results in both human and animal studies, showing an impaired production of antibodies and antibody response [3]. Other studies however, have reported no effect on humoral immunity and significant reduction of type-1 cytokine production [13].

It should be noted that the whole blood cultures were done in the presence of EDTA, which binds most soluble zinc (>99%) in the culture. The means that the lower IL-6 production in the zinc deficient infants is due to the pre-existent effects of zinc deficiency. The results suggest that cytokine production is reduced not only because of a reduced WBC count, but also because of impaired cytokine production by each leucocyte.

The fact that there was no clear relationship between iron status and immune function in this study, can probably be attributed to the large physiological changes in iron status indicators during the first year of life.

Thus, this study has shown that vitamin A deficiency has marked effects on the cell-mediated immune response, with decreased *ex vivo* type-1 cytokine production after stimulation, and with increased *in vivo* macrophage activity. Zinc deficiency also has distinct effects on immune function, resulting in reduced circulating leukocytes, and reduced *ex vivo* cytokine production after stimulation. The implication of these findings is that when infants are micronutrient deficient, it is likely that immune function is also impaired. The extent to which either of these arms is impaired appears to be determined by the specific micronutrient deficiency. These changes probably bear direct relevance for susceptability to infections.

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Growth Patterns.

Most people consider growth as a slow but continuous process, the end result of millions of unsynchronised cell replications. This idea was challenged however by Lampl et al. who measured 31 infants, aged 4 to 15 months, daily or weekly for almost 2 years. They reported that linear growth of these infants occurred in short bursts, followed by long periods of stasis. On average, the infants only grew on 1 out of 11 days, whereas on the other days the infants did not grow or even shrank. The mean increment on the day of growth however was 0.95 cm. Variability was high with some infants not growing for a long time (up to 63 days) and one child growing an astonishing 1.65 cm in one day. They propose a model of "saltation and stasis", although they do not offer an explanation for their findings and cannot specify which (hormonal) signal would be responsible for the synchronisation of cell division. Of course, their model has been challenged by others, who argue that a continuous model of growth fits the data just as well. By using very short measurement intervals the authors might have inadvertently introduced extra noise or error. Also, infants do not always cooperate. By comparison, daily measurements of growth in rabbits did not show any saltatory pattern. Karlberg et al proposed another model of human growth, but on a larger time scale, distinguishing three different phases of growth. The first exponential growth phase is during infancy and is nutrient dependent. This is followed by an almost linear growth phase during childhood in which growth hormone is important. Finally during puberty, growth can be modelled as a logistic function, driven by sex hormones. The overall growth curve is then the cumulative result of these three phases. A delay in the onset of the childhood phase seems to be the main determinant of early growth faltering. However, Karlberg et al could not identify any causal factor in neither Swedish nor Pakistani infants, which caused this delay. Also, growth faltering in Indonesia appears to start within the first months of life. Human growth remains a fascinating phenomenon.

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CHAPTER 4

Iron and Zinc Supplementation in Indonesian Infants: Effects on Micronutrient Status and Growth.

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Submitted for publication.

information on interactions between iron and zinc when supplemented together in infancy.

Deficiencies of micronutrients often coexist and have independent as well as interacting effects (Whittaker 1998; Dijkhuizen et al. 2001). Deficiencies can share a common etiology, but can also aggravate each other. In populations with a marginal iron and zinc status, the negative effects of high intake of iron (e.g. with supplementation) on zinc uptake and vice versa are important issues. Currently, in view of the serious consequences of iron deficiency in children, large-scale iron supplementation of under five children is being contemplated to combat iron deficiency. In this respect however, the possible negative effects on zinc status are a major concern.

The aim of this study is to investigate whether the supplementation of iron, zinc, or iron and zinc combined can improve iron status and zinc status and reduce the prevalence of deficiencies of iron and zinc, and whether supplementation can prevent growth faltering in the first year of life. Also, the interaction between iron supplementation and zinc supplementation and possible negative effects of supplementation on iron status and zinc status are investigated. This study collaborated in the UNICEF-coordinated Multi-Country Iron and Zinc Intervention Trials Collaborative Group.

SUBJECTS AND METHODS

Study design and location.

The study was designed as a randomised, double-blind, placebo-controlled supplementation trial in infants aged 4 mo at recruitment. Supplementation, not very different from the RDA (FAO/WHO 1988), was given 5 days per week for 6 mo by trained village health volunteers. Four groups of infants were supplemented with a syrup containing either iron (10 mg/day), zinc (10 mg/day), iron+zinc (10 mg of each/day) or placebo. Supplements were made by a local pharmaceutical company in cooperation with UNICEF-Jakarta. The study was carried out in a rural area of Bogor District, West Java, Indonesia between October 1997 and March 1999.

Subjects and procedures.

Eligible infants were identified by the village health volunteers, and the mothers invited to participate in the study. Mothers were informed of the procedures and purpose of the study. After written informed consent was given by the mother, infants were assessed anthropometrically, and a short history concerning socio-economic status, dietary and lactation habits, and health of the mother and infant was taken.

Exclusion before recruitment was on grounds of chronic or severe illness, severe clinical malnutrition, or congenital anomalies. Infants were assigned to one of the four supplementation groups on basis of individual randomization, using a block randomized group allocation list, which was computer generated before the study was commenced.

Supplementation was double-blind, the supplements were coded with a letter at production, and the code-allocation was safe-kept at the Wageningen University in The Netherlands. The codes were not known at the study site in Indonesia. The code was made known only after all subjects had completed the trial.

At recruitment, every subject received a personal bottle with a dosing syringe, labeled with the subject's name, subject number, health volunteer's name, and date. The bottles were kept safe by the health volunteer who gave the supplement each day, also to prevent accidental intoxication or overdosing. Supplements (2 mL of syrup) were given 5 days per week, and every dose given was tallied. Bottles were weighed before emission, replaced every month with a new bottle, and weighed again after return to estimate the dose given to the infant as a measure of compliance.

At the monthly follow-up, the infant was assessed anthropometrically, and a short history concerning health, diet and lactation and possible adverse effects was taken. After 6 mo of supplementation, in addition to the usual follow-up procedure, a blood sample was taken of the infant for biochemical assessment of nutritional status, as well as a stool sample to check for parasite infestation. All infants with a hemoglobin concentration of <110 g/L were given iron supplementation treatment.

Methods.

Anthropometry included measurement of weight, height and mid-upper-arm circumference by trained anthropometrists using standard methods. In addition, knee-heel length was measured with a knemometer. Z-scores for weight and height (weight-for-age WAZ; height-for-age, HAZ; and weight-for-height, WHZ) were calculated with EPI-Info 6.02, using WHO recommended growth curves (WHO Working Group 1986).

A non-fasting 5 mL venous blood sample was taken from the infants. A closed-tube heparanized vacuum system was used to avoid zinc contamination (Becton and Dickinson. Leiden, The Netherlands). Blood samples were immediately stored at 4°C to prevent microhemolysis and separated within 5 h. Plasma samples were aliquoted and stored at -30°C until analysis.

Hemoglobin concentrations were measured by the standard cyanoblue method (Humalyzer, Germany). Plasma zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace-element free procedures, as described in an earlier paper (Dijkhuizen et al. 2001). The CV (10% duplicate analysis and pooled control samples) for zinc analyses was <5%. Ferritin and insulin-like growth factor 1 (IGF-1) were measured using commercial ELISA-kits (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. C-reactive protein (CRP) was measured using immunoturbidimetric techniques at the Northern Ireland Centre for Diet and Health, University of Ulster, Northern Ireland (Cobas Fara analyzer, Roche Products, Welwyn, UK). The CV for the ferritin, IGF-1 and CRP assays was <10%. Plasma CRP concentrations were analyzed to assess occurrence of the acute-phase reaction, which will lower plasma concentrations of zinc and raise plasma concentrations of ferritin (Filteau and Tomkins. 1994). Stool samples were screened for the most commonly occurring parasites (hookworm, Trichuras, and Ascaris) by an experienced microscopist.

Ethical approval.

The protocol was approved by the ethical committee of the National Health Research and Development Institute of Indonesia and by the ethical committee of the Royal Netherlands Academy of Arts and Sciences.

Statistical analysis.

Data was checked for normal distribution using the Kolmogorov-Smirnov test of normality. Plasma concentrations of ferritin and zinc were transformed to logarithms before statistical analysis. Differences in prevalence were tested with Pearson's chi-square test, differences between infants who did not complete the study and those who did were tested with Student's t-test, and differences between baseline and end-point anthropometry were tested for each supplementation group with paired t-tests.

Differences for biochemical indicators between supplementation groups were analyzed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Plasma concentrations of CRP were used as covariate in the analysis of plasma concentrations of ferritin and zinc, to control for the effects of the acute phase response on plasma concentrations of both ferritin and zinc. Differences between the supplementation groups for anthropometry at recruitment (0 mo), after 3 mo, and 6 mo of supplementation were analysed with repeated measurements multivariate ANOVA statistics. For HAZ, sex (2 groups) and HAZ at recruitment (divided into 3 classes: HAZ<-2.0; -2.0≤ HAZ< -1.0; HAZ≥ -1.0) were used as additional between-subject factors to correct for possible effects of differences among groups at baseline. For the analysis of WAZ and WHZ over time, only sex was added as between-subject factor.

When the overall F-test was significant, differences between the groups were further investigated with post-hoc multiple comparisons for ANOVA, and in a general linear model for ANCOVA. Statistical analysis was carried out with EPI-Info, Version 6.04b (CDC, Atlanta GA) and SPSS 7.5.2 (SPSS Inc, Chicago, IL) software packages.

RESULTS

In total 478 infants were recruited from six adjacent villages in rural West Java, Indonesia. In total 360 infants completed the supplementation trial, and of 348 infants a complete blood sample was collected.

Of the recruited infants, 26 (5%) never came back for follow-up and are considered non-sincere recruits. A further 92 infants (19%) dropped-out during the study for various reasons (non-cooperation 13%, moving house 5% and mortality 3 cases (<1%), **Fig. 1**). The infants who dropped out did not differ from the infants who completed the trial for any of the characteristics at recruitment (Student's t-test). However, the drop-out in the group receiving the combination of iron and zinc was significantly higher than that in the other groups (36% vs. 18% - 22%, P <0.01, chi-square).

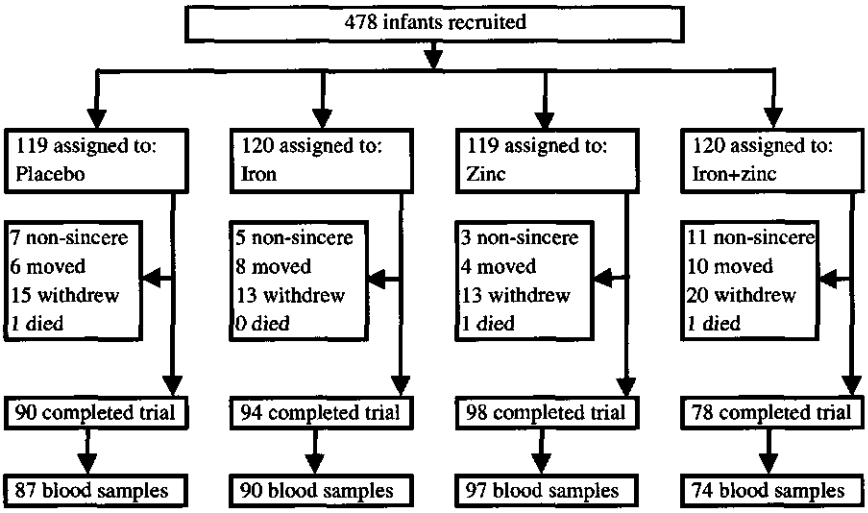

Figure 1. Trial profile.

The mean age of the infants at recruitment was 4.2 mo, and there were no significant differences between the groups at recruitment for any of the parameters measured (chi-square and ANOVA, **Table 1**). The mean WHZ of the infants were positive, whilst the mean HAZ were negative, indicating that especially length growth is suboptimal in these infants. Of the infants, 25 (5%) had a HAZ of < -2, and only one infant had a WHZ < -2 at recruitment. Mean knee-heel length (± SD) at recruitment was 16.4 (± 0.9) cm and did not differ among the groups (ANOVA).

Monthly compliance was measured by comparing the weights of the bottles before emission with the weights after return, and for every subject an overall compliance was calculated. Mean overall compliance (± SD) was 86% (± 16%) of the total intended dose, and was similar for all groups (chi-square).

At the end of the 6 mo supplementation period, the infants had a mean age (± SD) of 10.1 (± 0.6) mo. The hemoglobin concentrations of the infants who received iron alone or in combination with zinc, were significantly higher than of the infants who received placebo (**Table 2**, $P < 0.01$ and $P < 0.05$ respectively, ANOVA). Also, the hemoglobin concentrations of the infants receiving iron alone, were significantly higher than of the infants receiving zinc alone (**Table 2**, $P < 0.01$, ANOVA). The proportion of infants with anemia (hemoglobin concentration <110 g/L) in the iron group was significantly lower than in placebo and zinc groups ($P < 0.01$, chi-square) but also lower than in the iron+zinc group ($P < 0.05$, chi-square, **Table 2**). Furthermore, the prevalence of anemia in the iron+zinc group was significantly lower than in the placebo and zinc groups ($P < 0.05$, chi-square, **Table 2**). Plasma ferritin concentrations were significantly higher in the iron and iron+zinc supplementation groups as compared to both the placebo and zinc groups ($P < 0.01$, ANCOVA with CRP as covariate). The proportion of infants who were iron deficient (hemoglobin concentration < 110 g/L and

TABLE 1. General characteristics of the supplemented infants at recruitment.[1]

	Placebo	Iron	Zinc	Iron+zinc
n	119	120	119	120
Boys : Girls (n)	53 : 66	62 : 58	71 : 48	64 : 56
Age (mo)[2]	4.2 (± 0.4)	4.2 (± 0.5)	4.2 (± 0.5)	4.2 (± 0.5)
Mean Z scores[2]				
Weight-for-age	-0.14 (± 0.88)	-0.06 (± 0.93)	0.05 (± 0.86)	-0.06 (± 0.85)
< -2.0 (n)	*2*	*2*	*0*	*1*
Height-for-age	-0.88 (± 0.77)	-0.89 (± 0.82)	-0.70 (± 0.72)	-0.91 (± 0.78)
< -2.0 (n)	*6*	*7*	*4*	*8*
Weight-for-height	0.67 (± 0.84)	0.77 (± 0.83)	0.72 (± 0.82)	0.80 (± 0.85)
< -2.0 (n)	*1*	*0*	*0*	*0*
Knee-heel length (cm)[2]	16.3 (± 0.9)	16.4 (± 1.1)	16.6 (± 0.8)	16.4 (± 0.9)

[1] No differences between the groups.
[2] Mean (± SD).

TABLE 2. Indicators of micronutrient status in infants after 6 mo of supplementation with iron and zinc.

Supplementation group	Placebo	Iron	Zinc	Iron+zinc
n	87	90	97	74
Hemoglobin concentration (g/L)[1]	106 (± 11)	115 (± 10) [3,4]	106 (± 11)	110 (± 11) [5]
Proportion anemic (%)[2]	*66*	*28* [3,4,6]	*62*	*46* [5,7]
Plasma ferritin concentration (μg/L)[1]	14.3 (8.1-25.3)	36.8 (19.1-61.2) [3,4]	12.0 (6.0-21.2)	26.6 (16.9-41.7) [3,4]
Proportion iron deficient (%)[2]	*30*	*3* [3,4]	*35*	*8* [3,4]
Plasma zinc concentration (μmol/L)[1]	13.0 (10.7-15.3)	13.5 (11.4-15.3)	16.1 (13.4-20.3) [3,8]	15.0 (13.2-18.4) [3,8]
Proportion plasma zinc < 10.7 μmol/L (%)	*24*	*17*	*9* [3]	*8* [3]

[1] Means (± SD) or medians (IQR)

[2] Anemia is defined as hemoglobin concentration <110 g/L, and iron deficiency as anemia combined with a plasma ferritin concentration < 12 μg/L

[3] Different from placebo group, P <0.01

[4] Different from zinc group, P <0.01

[5] Different from placebo group, P <0.05

[6] Different from iron+zinc group, P <0.05

[7] Different from zinc group, P <0.05

[8] Different from iron group, P <0.01

plasma ferritin concentration <12 μg/L) was significantly lower in the iron and iron+zinc supplementation groups (P <0.01, chi-square, **Table 2**). Plasma zinc concentrations were significantly higher in the zinc and iron+zinc supplementation groups than in the placebo and iron groups (P <0.01, ANCOVA with CRP as covariate, **Table 2**). The proportion of infants with a low plasma zinc concentration (< 10.7 μmol/L) was significantly lower in the zinc and iron+zinc groups than in the placebo group (P <0.01, chi-square, **Table 2**).

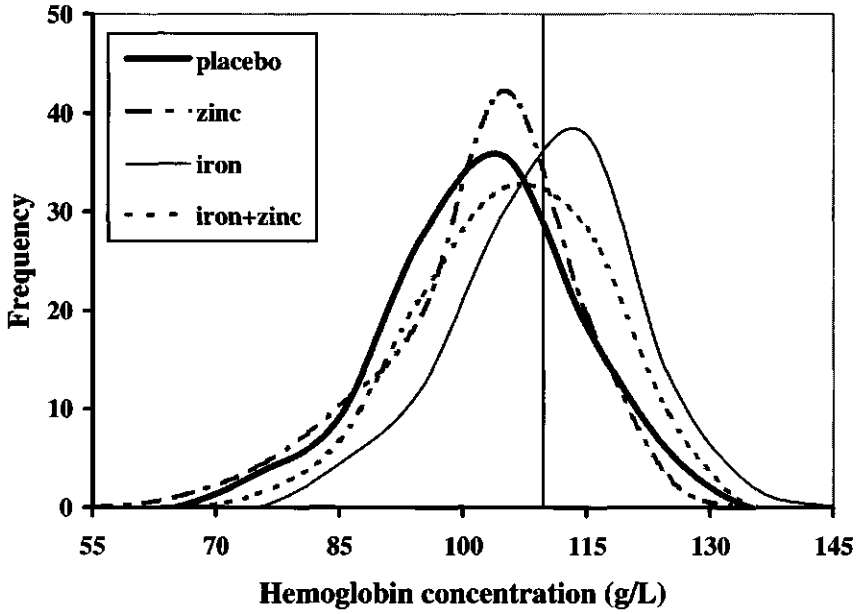

Figure 2. Hemoglobin concentration frequency distribution curves (smoothed) of infants supplemented with iron and zinc.

The effect of iron and zinc supplementation on hemoglobin and plasma zinc concentration is clearly illustrated by the frequency distribution curves (**fig. 2 and fig. 3**). For hemoglobin concentrations, the iron group curve is clearly shifted to higher hemoglobin concentrations as compared to the placebo and zinc group curves, whilst the iron+zinc group curve is intermediate between the iron and placebo group curves. The distribution curves for zinc concentration are somewhat less smooth, and the effect of supplementation is clearest when comparing the areas under the curve below a plasma zinc concentration of <10.7 μmol/L for the different groups. The placebo group curve has the largest area with low plasma zinc concentrations, whereas both the zinc and iron+zinc group curves have the smallest areas below the cut-off for zinc deficiency. Interestingly, the zinc group curve seems to be shifted more towards higher plasma zinc concentrations than the iron+zinc group curve.

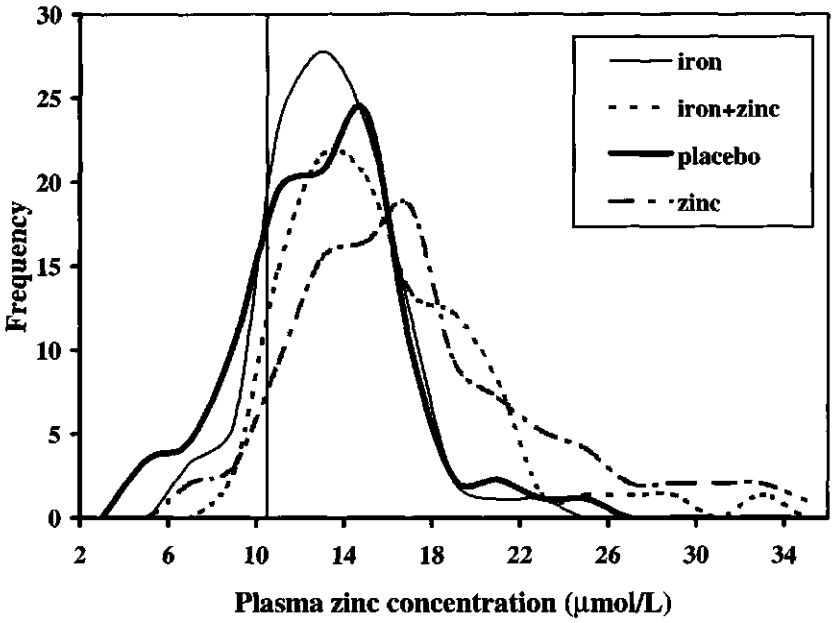

Figure 3. Plasma zinc concentration frequency distribution curves (smoothed) of infants supplemented with iron and zinc.

Anthropometry Z-scores of the four supplementation groups were compared at recruitment (0 mo), after 3 mo of supplementation (3 mo) and at the end of the 6 mo of supplementation (6 mo). All groups had a significant decrease in HAZ (**fig. 4**), WHZ (**fig. 5**) and WAZ (all $P < 0.01$, paired t-test). For comparison of the HAZ changes over time between the different supplementation groups, sex and HAZ at recruitment were included in the analysis as between subject factors. There was no effect of either iron or zinc supplementation on changes in HAZ over time (**fig. 4**). The baseline factors did not interact significantly with the height changes per group. Differences in changes in WHZ over time among the groups were analyzed, taking sex into account. There was also no effect of either iron or zinc supplementation on ponderal growth (**fig. 5**). There was no significant interaction of sex on changes in WHZ per supplementation group. After 6 mo of supplementation, stunting (HAZ < -2.0) was found in 24% of the infants in the placebo group, 19% of the iron group, 18% of the zinc group and 21% of the iron+zinc group. There was no difference in prevalence of stunting between the groups (chi-square).

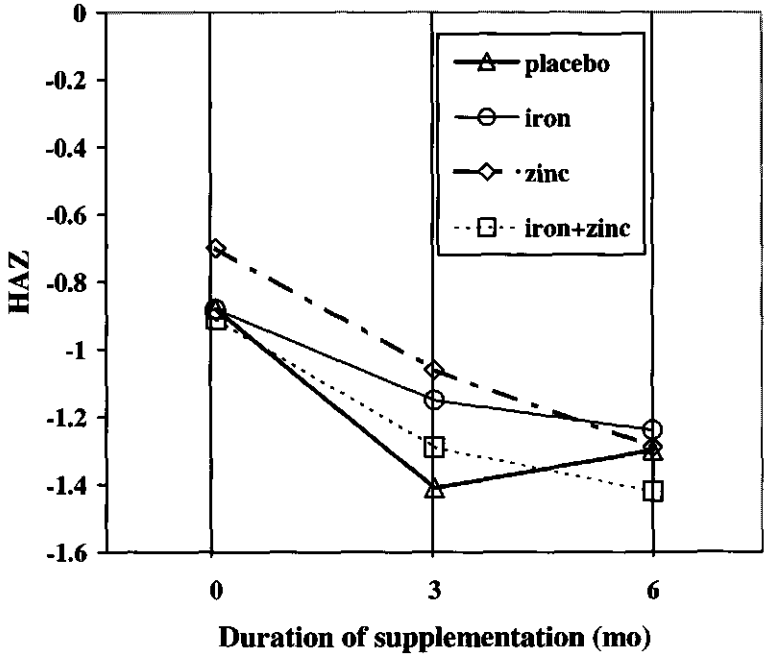

Figure 4. Linear growth , expressed as Z scores for height for age (HAZ), of infants supplemented with iron and zinc.

The knee-heel length after 6 mo of supplementation did not differ among the groups (mean (± SD) for the placebo group: 18.6 (± 0.9) cm; for the iron group: 18.8 (± 1.0) cm; for the zinc group: 18.9 (± 0.9) cm; for the iron+zinc group18.7 (± 0.9) cm respectively, ANOVA). Plasma IGF-1 concentrations were measured after 6 mo of supplementation as an indicator for growth activity. There were no significant differences among the supplementation groups (overall mean (± SD): 20.5 (± 16.3) μg/L, ANOVA).

Parasitological examination of stool samples gave an infection rate of <5%, and was not different among the groups, hence parasite infestation was not considered a major factor in the outcome of the study.

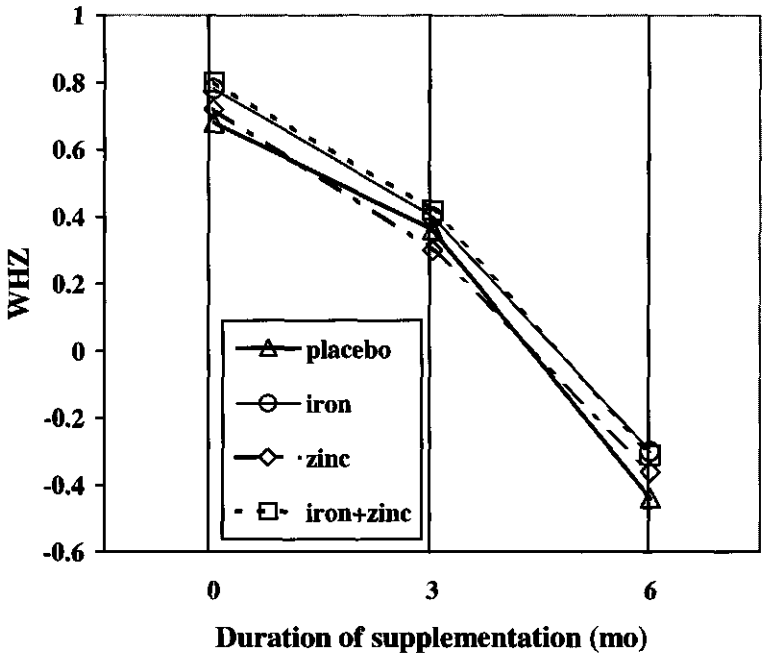

Figure 5. Ponderal growth, expressed as Z scores for weight for height (WHZ) of infants supplemented with iron and zinc.

DISCUSSION

This study shows that anemia, iron deficiency, and zinc deficiency are very prevalent in these infants, and that supplementation with iron and zinc is very effective to combat these deficiencies. The combined supplementation of iron and zinc was found to be slightly less efficient than either supplement alone, but still effective in reducing both iron deficiency and zinc deficiency.

An important finding is that iron supplementation alone does not seem to have negative effects on zinc status, as the plasma zinc concentrations in the iron supplementation group are very similar and certainly not lower than those of the placebo group. Furthermore, the prevalence of zinc deficiency is not increased in the iron group. This indicates that iron supplementation in young infants given daily in a dosage (10 mg/day), not very different from the RDA (8.5 mg/day), will not imperil zinc nutriture. Also, zinc supplementation given alone does not affect iron status to any extent. It would seem that in these infants, supplementation of iron as well as zinc has the largest benefits, reducing the prevalence of both iron as well as zinc deficiency to less than one third of the prevalences in the placebo group. However, for the combined supplement of iron and zinc, the effectiveness appears to be somewhat less than for either supplement alone, as the combined supplement did not reduce anemia as much

as did supplementation with iron alone. Also the plasma zinc concentration distribution curve of the group supplemented with zinc alone seems to be shifted more effectively towards higher plasma zinc concentrations.

A combined supplement seems the most desirable in these infants considering the high prevalence of anemia (66%), iron deficiency (30%) and zinc deficiency (24%) in this population. It is cause for concern that the drop-out in the iron+zinc supplementation group is almost double that of the other supplementation groups. This could indicate an acceptability problem of the combined supplement. Gastro-intestinal complaints are the most likely cause of this higher drop-out, as minor complaints such as bad taste of the supplement would have affected compliance also, but this was the same in all groups. Perhaps this acceptability problem is in part due to the composition of the supplement used, with iron and zinc as sulphate salts in a 1:1 weight ratio. Sulphate salts are the least well tolerated, as they are more likely to cause gastro-intestinal complaints, and also an 1:1 weight ratio of iron and zinc might not be the most favorable. This study was not designed to investigate the optimal composition of the supplements, but this area certainly merits further attention.

The anthropometrical assessment of these infants showed a striking decrease in Z-scores from the age of 4 mo to the age of 10 mo. Although supplementation with both iron and zinc was very effective in decreasing the prevalence of deficiencies of iron and zinc, there was no effect of supplementation of either iron or zinc on growth. Even though supplementation was started before significant growth impairment had occurred, supplementation with iron, zinc or both was not able to prevent progressive stunting. After 6 mo of supplementation, 21% of the infants were stunted. In all groups, growth was impaired to the same extent and followed the same pattern. The lack of effect of supplementation of iron and zinc on growth is further confirmed by the lack of effect on plasma IGF-1 concentration, which is an indicator of growth activity. There were no differences in growth activity among the groups.

Ninh et al. (1996) showed that zinc supplementation of Vietnamese children increased plasma IGF-1 concentrations as well as growth performance, but only stunted children were supplemented. In Ethiopia, growth of both stunted and non-stunted infants benefited from zinc supplementation, although the effect in stunted infants was larger (Umeta et al. 2000). Iron intake in Ethiopia however is very high, possibly creating exceptional conditions for zinc nutriture. Rosado et al. (1997) found no effect of either zinc or iron supplementation on growth in Mexican children, aged > 1 y. There was a small but significant effect of zinc supplementation on morbidity, but no interaction between iron and zinc. From a meta-analysis of published randomized trials, Brown et al. (1998) conclude that zinc supplementation had a significant but small effect on growth, especially in stunted children. In the present study, the infants were not selected for stunting, and supplementation was started at an age when stunting is not yet prevalent. This study aimed to prevent stunting, and not to improve growth in already stunted infants.

In conclusion, the results of this study show that even though there is a high prevalence of deficiencies of iron and zinc, combating these deficiencies is not sufficient to allow optimal growth in these infants. There must be additional underlying

factors in the diet or circumstances of these infants that effectively impair growth. In view of the detrimental effects of iron deficiency on development, and of zinc deficiency on morbidity and possibly growth, supplementation of iron and zinc to combat the high prevalence of these deficiencies can play an important role in improving infant health. This study shows that supplementation with a combination of iron and zinc can be safe and effective.

ACKNOWLEDGEMENTS

We would like to thank all the mothers and the infants, and the health volunteers who participated in this study, and we are grateful for the enthusiastic support we received from Dr. Hendra, Dr. Anni and their staff at the Puskesmas Situ Udik. Furthermore we would like to thank the field team of Puslitbang Gizi, and the laboratory staff from the Nutrition Research and Development Centre, Bogor for their untiring efforts. Also we would like to thank D. Thurnham, C. Northrop-Clewes and J. Coulter from NICHE, University of Ulster for their help with the analysis of CRP. Lastly we would like to thank P. Wieringa and H. Wieringa-Brants for providing invaluable logistic support. Financial support for this study was received from the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), Ter Meulen Fund (Royal Netherlands Academy of Arts and Sciences), and UNICEF Jakarta.

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Yellow Gecko Eyes.

In humans, retinol binding protein (RBP) is the main transport protein of retinol. During evolution, several forms of RBP have emerged to allow optimal function as transport protein of retinol in either the plasma or intracellularly. However, RBP can fulfil other functions also. Recently, it was shown that the lens of the eye of diurnal geckos contain up to 12% of a protein very similar to cellular RBP type I. Interestingly, this protein does not have retinol as ligand but 3,4 didehydroretinol instead. The 3,4 didehydroretinol–RBP complex gives the lens a yellow colour, and absorbs short-wave radiation, thereby protecting the retina. Retinol, in contrast to 3,4 didehydroretinol, is unsuitable as ligand as it fluoresces in the range of visible light and is not photostable. The authors speculate that during evolution, nocturnal geckos have changed to diurnal habits, resulting in exposure to often intense ambient light. Unlike humans, geckos cannot regulate light intensity by changing the diameter of the pupil, hence they need other ways to protect the retina. Mutations resulting in the expression of cellular RBP-I with 3,4 didehydroretinol as ligand in the lens may thus have conferred evolutionary benefits. In this respect it is important to note that 3,4 didehydroretinol is found in the lenses of diurnal, but not in nocturnal geckos. As 3,4 didehydroretinol normally doesn't occur in terrestrial vertebrates, it is probably converted from retinol in the eye of diurnal geckos especially for this purpose. Interestingly, 3,4 didehydroretinol is also used in the modified relative dose response test, which measures liver retinol stores.

Werten PJL, Röll B, Van Aalten DMF, De Jong WW. Proceedings of the National Academy of Sciences (USA). 2000;97:3282-87.

CHAPTER 5

Iron Supplementation Can Induce Vitamin A Deficiency in Infants with Marginal Vitamin A Status.

Frank T. Wieringa, Marjoleine A. Dijkhuizen, Clive E. West, David I. Thurnham, Muhilal, Jos W.M. van der Meer.

Submitted for publication.

ABSTRACT

Background. Deficiencies of iron and vitamin A are still very prevalent worldwide. Supplementation, mostly with single micronutrients, is widely used to combat these deficiencies. However, various micronutrient deficiencies often occur concurrently, and there are many interactions between micronutrients. This study investigates the interactions between 3 important micronutrients, iron, vitamin A and zinc, when supplemented alone or combined.

Methods. In a randomised, double-blind, placebo-controlled supplementation trial, 387 Indonesian infants, aged 4 months, were supplemented daily for 6 months with iron (10 mg), zinc (10 mg), β-carotene (2.4 mg), iron+zinc (10 mg each), zinc+β-carotene (10 mg +2.4 mg) respectively, or placebo. From the 271 infants who completed the trial, complete data on micronutrient status, using haemoglobin, ferritin, retinol, zinc and the modified relative dose response (MRDR, a measure of liver retinol stores), was available from 256 infants.

Findings. Iron supplemented infants had significantly lower plasma retinol concentrations, and thus significantly higher prevalence of vitamin A deficiency than all other groups. In contrast, the MRDR of the iron supplemented infants indicated higher liver stores of vitamin A. Furthermore, iron supplementation improved iron status while zinc supplementation improved zinc status but β-carotene supplementation failed to significantly improve vitamin A status.

Interpretation. In this study, iron supplementation of a population marginally deficient for vitamin A led to a significant increase in the prevalence of vitamin A deficiency. The finding of increased vitamin A liver stores after iron supplementation suggests that there is a redistribution of retinol. Therefore, supplementation of infants with iron should be accompanied by measures to improve vitamin A status.

INTRODUCTION

Iron deficiency and its associated anaemia, as well as vitamin A deficiency are still very prevalent worldwide, with children and pregnant women being the most vulnerable groups. Anaemia and iron deficiency in children can lead to delayed psychomotor development and impaired health (1). Vitamin A deficiency is associated with a markedly higher morbidity of infectious diseases and mortality, and severe deficiency leads to xerophthalmia and blindness (2). Zinc deficiency leads to growth impairment, and zinc supplementation has been shown to decrease morbidity and mortality of infectious diseases in children (3-5). In many countries, major efforts are being made by governments supported by international organisations, to reduce micronutrient deficiencies. For example, high dose vitamin A supplements are globally distributed to children under five years of age, and WHO/UNICEF recommend supplementation with iron for infants from 6 months onwards in countries with a high prevalence of iron deficiency anaemia (6).

Micronutrient deficiencies often occur together in populations, because the same dietary patterns and socio-economic factors are associated with deficiency of many different micronutrients. In many developing countries, diets are mostly cereal-based, low in animal products and high in phytate, thus giving a high risk of micronutrient deficiencies. In Indonesia, deficiencies of iron, vitamin A, and zinc are prevalent and often concurrent (7).

Interactions between iron, vitamin A and zinc nutriture are of great consequence, especially in the context of micronutrient supplementation. Iron supplementation is widely used to combat iron deficiency, however high iron intake is known to impair zinc uptake (8). Furthermore, vitamin A supplementation affects iron metabolism. It has been shown to decrease anaemia prevalence, and has a synergistic effect with iron supplementation in reducing anaemia (9). Provitamin A carotenoids, especially β-carotene are the most important sources of vitamin A in Indonesia, but absorption, especially from green leafy vegetables, is insufficient (10). Severe zinc deficiency can impair vitamin A status (11), and zinc supplementation may be able to improve uptake of β-carotene, its subsequent bioconversion to retinol and the mobilisation of vitamin A from body stores in populations with marginal zinc status (12).

This study investigated whether supplementation of micronutrients, alone or in combination, enhances or impairs status with respect to other micronutrients. Interactions between iron, β-carotene and zinc, as well as effectiveness of supplementation on improving micronutrient status were analysed. Special attention was given to iron and vitamin A as these micronutrients are included in many nutrition intervention programmes, but usually only given as single micronutrients.

SUBJECTS AND METHODS

Study design, subjects and procedures.

The study was a randomised, double-blind, placebo-controlled supplementation trial in infants aged 4 months at recruitment. The study was carried out in a rural area of Bogor District, West Java, Indonesia. Supplementation was given 5 days/week for 6 months by trained village health volunteers. Six groups of infants were supplemented with a sweet, strawberry flavoured, clear syrup (2 mL/day) and neutral tasting, brightly orange coloured corn oil (0.5 mL/day). Four syrup preparations contained ferrous sulphate, zinc sulphate, ferrous sulphate + zinc sulphate, or neither ferrous sulphate nor zinc sulphate. The oil contained either β-carotene or annato (both from Quest International, Naarden, The Netherlands), a permitted food additive with no provitamin A activity, but the same colour as β-carotene. The iron and zinc supplements were produced by a local pharmaceutical company (Kenrose Ltd., Jakarta, Indonesia) in cooperation with UNICEF-Jakarta. The infants received either iron (10 mg Fe/day), or zinc (10 mg Zn/day), or iron+zinc (10 mg of each/day), or zinc+ β-carotene (10 mg/day and 2.4 mg/day respectively), or β-carotene (2.4 mg/day) or placebo.

Eligible infants were identified by the village health volunteers, and the mothers invited to participate in the study. Mothers were informed of the procedures and purpose of the study. After written informed consent was given by the mother, infants were assessed anthropometrically, and a short history was taken. Infants were assigned to one of the six groups on basis of individual randomization, using a block randomised group allocation list, which was computer generated before the study was commenced.

Supplementation was double-blind, and the supplements were coded with a letter at production. The allocation code was kept safe at the Wageningen University until the study was finished.

At recruitment, each subject was allocated a personal bottle with a dosing syringe, labeled with the subject's name, subject number, and date. The bottles were kept safe by the health volunteer to prevent accidental overdosing. Bottles were weighed before allocation, replaced every month with a new bottle, and weighed again after return to estimate the dose given to the infant as a measure of compliance.

At each monthly follow-up, the infant was assessed anthropometrically, and a short history concerning health, diet, lactation and possible adverse effects of the supplements was taken. After 6 months of supplementation, a blood sample was taken from the infants for biochemical assessment of nutritional status. Five hours prior to the blood sampling, the infants received a dose of 3,4-didehydroretinol in corn oil (1.5 mg in 700 μL oil) for the modified relative dose response (MRDR) test. The MRDR test uses the ratio of 3,4-didehydroretinol to retinol as an indicator of liver stores of vitamin A (13). When vitamin A liver stores are low, more 3,4-didehydroretinol appears in the blood relative to retinol concentrations. A ratio of > 0.06 of the concentrations in plasma is considered indicative of insufficient stores of vitamin A in the liver (13). All infants with a haemoglobin concentration of <110 g/L were given iron supplementation treatment upon completion of the trial. The protocol was

approved by the ethical committee of the National Health Research and Development Institute of Indonesia and by the ethical committee of the Royal Netherlands Academy of Arts and Sciences.

Measurements and analysis.

A non-fasting venous blood sample (5 mL) was taken from the infants into heparinized vacuum tubes (Becton and Dickinson. Leiden, The Netherlands). Blood samples were immediately stored at 4°C to prevent microhaemolysis and separated within 5 hours. Plasma samples were aliquoted and stored at –30 °C until analysis.

Haemoglobin concentrations were measured the same day by a standard cyanoblue method (Humalyzer, Germany). Plasma zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace-element free procedures. The CV (10% duplicate analysis and pooled control samples) for zinc analyses was <5%. Plasma retinol, carotenoids and 3,4-didehydroxyretinol concentrations were measured by HPLC (MilliporeWaters, Harrow, Middlesex, UK) (14). 3,4-Didehydroxyretinol and retinol were measured in the same extract as carotenoids but using a different mobil phase (methanol:water 90:10 v/v). The CV (10% duplicate analysis and pooled control samples) for the retinol and carotenoid analysis was <10%. Ferritin concentrations were measured using commercial ELISA-kits (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. C-reactive protein (CRP) and α_1-acid glycoprotein (AGP) were measured using immunoturbidimetric techniques (Cobas Fara analyzer, Roche Products, Welwyn, UK). The CV for the ferritin, CRP and AGP assays was <10%. Plasma CRP and AGP concentrations were analysed to assess the acute-phase reaction, which lowers plasma concentrations of zinc and retinol, and raises plasma concentrations of ferritin (15). Plasma CRP concentrations rise early in the acute phase response, but normalise generally within two weeks. Plasma AGP concentrations rise more slowly than those of CRP, but stay elevated for up to two months after the initial acute phase response (16).

Statistical analysis.

Data were checked for normal distribution using the Kolmogorov-Smirnov test of normality. Plasma concentrations of ferritin and zinc were transformed to logarithms before statistical analysis. Differences in prevalence were tested with Pearson's chi-square test, differences between infants who dropped-out and infants who completed the study were tested with Student's t-test. Difference in compliance and plasma β-carotene concentrations was tested using the non-parametric Kruskal-Wallis test. Differences for age, biochemical indicators and anthropometry were analysed using analysis of variance (ANOVA) or analysis of covariance (ANCOVA). Plasma CRP and AGP concentrations were used as covariates in the analysis of plasma concentrations of retinol, ferritin, and zinc, to control for the effects of the acute phase response. When the overall F-test was significant, differences between the groups were further explored with post-hoc multiple comparisons for ANOVA, and in a general linear model for ANCOVA. In the general linear models, plasma retinol concentrations were transformed to logarithms as a variance stabilising measure.

Statistical analysis was carried out with the SPSS 7.5.2 (SPSS Inc, Chicago, IL) software package, and anthropometrical Z-scores were calculated with EPI-Info, version 6.04b (CDC, Atlanta, GA).

RESULTS

Between November 1997 and July 1998, 387 infants were recruited from six adjacent villages in Bogor District. Of these, 271 infants completed the supplementation trial and of 256 infants, a complete blood sample was collected (**Figure 1**). Of the recruited infants, 27 (7%) never came back for follow-up and were considered non-sincere recruits. A further 89 infants (23%) dropped-out during the study for various reasons (non-cooperation 18%, moving house 3% and mortality 5 cases (1%)). The infants who dropped out did not differ from the infants who completed the trial for any of the characteristics at recruitment. The drop-out among the groups ranged from 20% in groups receiving iron or zinc to 39% in the group receiving the combination of iron and zinc (chi-square, p=0.06). At recruitment, the mean age of the infants was 4.2 months [SD $\pm$ 0.4], and the male:female ratio was 206:181. Anthropometry was expressed as Z-scores, and the mean weight-for-age was –0.02 [SD $\pm$ 0.85]; the mean height-for-age was –0.88 [SD $\pm$ 0.77]; the mean weight-for-height was +0.82 [SD $\pm$ 0.82]. There were no differences among the groups for any of the baseline characteristics. The median overall compliance was 91% [IQR 76%-98%] of the total intended dose, and did not differ between the groups.

After six months of supplementation micronutrient status was assessed (**Table 1**). The mean age of the infants was 10.2 months [SD $\pm$ 0.5]. Infants receiving iron or iron+zinc had significantly lower plasma retinol concentrations than infants receiving placebo ($p<0.05$ and $p<0.001$ respectively). Haemoglobin concentrations were significantly increased in the infants receiving iron ($p<0.01$) but the increase just failed to reach significance for infants receiving iron+zinc ($p=0.052$). Plasma ferritin concentrations were highest in the group receiving iron, and also significantly higher in the iron+zinc supplemented group as compared to all other group ($p<0.001$ for both, ANCOVA controlling for CRP). Plasma zinc concentrations were significantly higher in the groups receiving zinc and zinc+ β-carotene ($p<0.001$, ANCOVA, controlling for CRP), and also higher but to a lesser extent in the group receiving iron+zinc ($p<0.05$). β-Carotene supplementation, either alone or in combination with zinc, significantly raised plasma β-carotene concentrations ($p<0.001$, Kruskal-Wallis), but failed to increase plasma retinol concentrations.

The effect of the three micronutrients supplemented on plasma retinol concentrations was further investigated using a general linear model, with CRP included as covariate. Supplementation with iron was associated with lower plasma retinol concentrations of 0.15 μmol/L ($p<0.01$). Neither zinc nor β-carotene supplementation significantly affected plasma retinol concentrations.

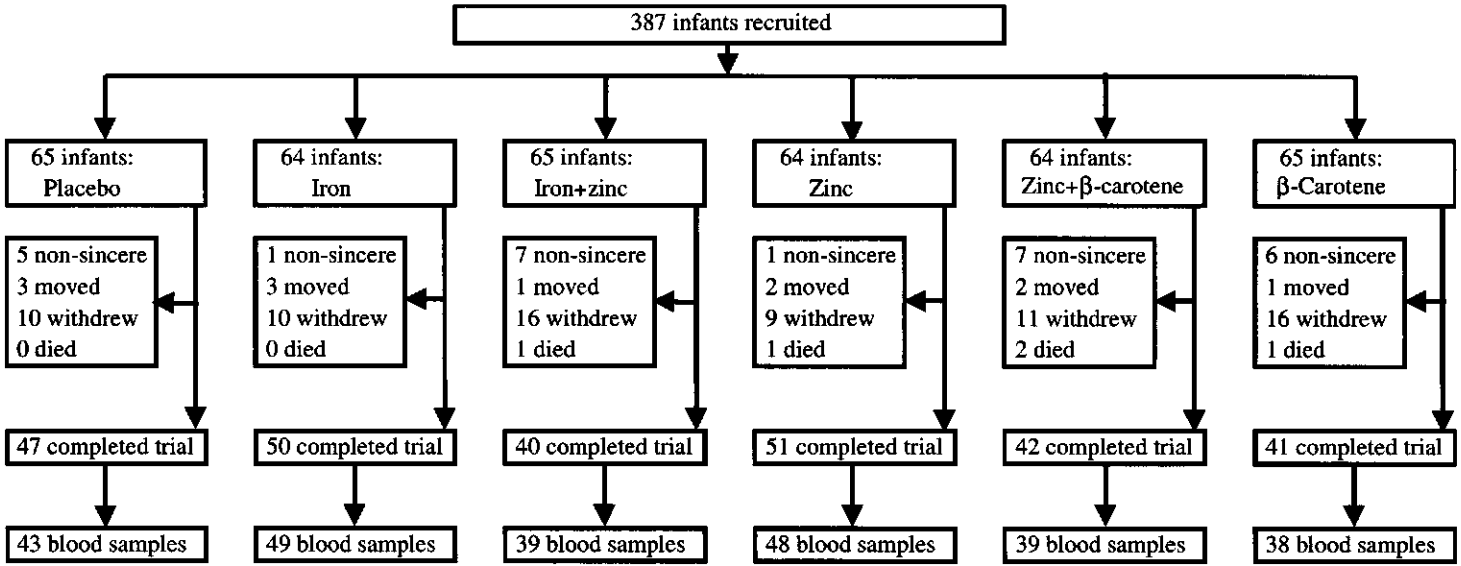

Figure 1. **Trial Profile**

In the placebo group, the prevalence of micronutrient deficiencies was high (**Figure 2**): 54% had vitamin A deficiency (plasma retinol concentration <0.70 μmol/L), 49% were anaemic (haemoglobin concentration <110 g/L), 26% had iron deficiency anaemia (anaemia and plasma ferritin concentration <12 μg/L), and 14% had zinc deficiency (plasma zinc concentration <10.7 μmol/L). However, the prevalence of vitamin A deficiency was significantly higher in the infants receiving iron or iron+zinc supplementation ($p<0.01$), 20% and 26% higher respectively compared to the placebo group. In contrast, the prevalence of insufficient vitamin A liver stores, as indicated by a MRDR >0.06, was lowest in the iron and iron+zinc groups ($p<0.01$; 30% and 32% respectively) than in the placebo group. Furthermore, the prevalence of anaemia and iron deficiency anaemia were lowest in the groups receiving iron and iron+zinc ($p<0.01$). Neither the reduction in the prevalence of vitamin A deficiency in the groups receiving β-carotene or zinc+β-carotene nor the reduction in the prevalence of zinc deficiency in the groups receiving zinc, iron+zinc and zinc+ β-carotene were large enough to be statistically significant.

The effect of iron supplementation on vitamin A status is clearly illustrated by the relationship between plasma retinol concentration and MRDR (**figure 3**). There is a strong linear relationship between plasma retinol concentrations and the MRDR for the groups not receiving iron ($r= -0.69$, $p<0.001$, Pearson correlation), with the infants with a low plasma retinol concentration having a high MRDR (indicative of depleted vitamin A liver stores). However, the groups receiving iron and iron+zinc supplements clearly form a separate subgroup, placed outside this relationship ($r=0.08$, $p>0.1$). In these subjects, in contrast to the infants not receiving iron or iron+zinc, a low plasma retinol concentration is accompanied by a low MRDR ($p<0.001$, chi-square).

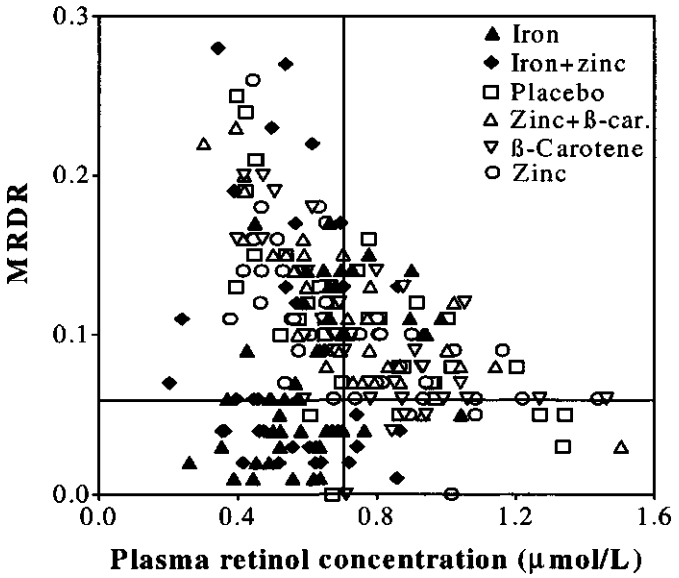

Figure 3. Relationship between the modified relative dose response and plasma retinol concentrations in infants supplemented with various combinations of micronutrients.

DISCUSSION

We find that a daily supplement of 10 mg of iron, which is not very different from the RDA, markedly reduces plasma retinol concentrations and increases the prevalence of vitamin A deficiency in infants. The lower MRDR in the iron supplemented infants, indicating increased hepatic stores of vitamin A, would suggest that iron supplementation redistributes vitamin A from plasma to liver. Such findings have not been reported previously. For example, our group reported that iron supplementation had no effect on plasma retinol concentrations in pregnant women (9). However, only 10% of the pregnant women were vitamin A deficient, and they were supplemented for only 8 weeks. No effect of either iron or zinc supplementation on plasma retinol concentrations was found in children in Mexico although plasma retinol concentrations were found to be increased six months after supplementation had stopped (17).

It is cause for concern that iron supplementation leads to plasma retinol concentrations below cut-off levels for vitamin A deficiency as low plasma retinol concentrations are directly related to the consequences of vitamin A deficiency (18). Although in the present study retinol liver concentrations were increased after iron supplementation, a reduced mobilisation of retinol from the liver to the plasma will induce a state of vitamin A deficiency. Thus iron supplementation, by inducing vitamin A deficiency, could increase the risk of morbidity and mortality from infectious diseases in infants. This would be especially important in populations where vitamin A status is already marginal.

An alternative explanation for the reduction of plasma retinol concentrations as a result of iron supplementation may be that vitamin A requirements are increased because of accelerated erythropoiesis. However, such an explanation would not be consistent with the reduced MRDR, signifying increased retinol liver stores. Whether redistribution of retinol after iron supplementation only occurs in populations with a high prevalence of both iron deficiency and vitamin A deficiency remains to be investigated. However, this is of great importance because these are precisely the populations targeted by micronutrient supplementation programmes.

Observations of the interaction between vitamin A and iron metabolism can be obscured by the acute phase response, which leads to increased plasma ferritin concentrations and to decreased plasma retinol concentrations. In the present study, CRP was included in the analyses to control for these effects. The proportion of infants with plasma CRP concentrations >10 mg/L was not different among the supplementation groups. Furthermore, excluding the 32 infants with CRP levels >10 mg/L did not change the findings. Using a different acute-phase protein (AGP) as covariate, or as exclusion criterion (plasma AGP levels >1.2 g/L; excluded n=56) also gave identical results. Hence, the decrease in plasma retinol concentrations in the iron supplemented groups cannot be attributed to differences in the acute phase response.

How should we interpret the increase in vitamin A liver stores after iron supplementation? On the basis of the MRDR measurements, we surmise that there is a redistribution of retinol after iron supplementation. The MRDR method may have

certain limitations. Because the measurement is derived from the ratio of the concentration of 3,4-dihydroxyretinol to that of retinol, precision is less than that of the measurement of plasma retinol alone. However, since the effect of iron supplementation on the MRDR was highly significant, this limitation is not of concern here. Furthermore, the MRDR may be affected by factors other than the concentration of vitamin A in liver. In children with protein-energy malnutrition, the MRDR is less sensitive because the concentration of many plasma proteins is low. Thus the low concentration of retinol-binding protein in protein-energy malnutrition distorts the competitive binding between 3,4-dihydroxyretinol and retinol (19). As the infants in the present study were not malnourished, it may be assumed that the MRDR adequately reflects retinol liver stores.

Most studies, including those using animal models, on the interrelation between iron and vitamin A metabolism have focussed on the influence of vitamin A on iron metabolism (20), but not vice versa. This is because earlier studies in humans have shown that supplementation with vitamin A in addition to iron has been shown to reduce the prevalence of anemia more than the provision of iron supplements alone9. However, Rosales et al. showed that retinol distribution over various body compartments is dependent on iron status, with lower concentrations of vitamin A in the liver of iron deficient rats than in food-restricted control rats (21). On the other hand, Jang et al. showed that iron deficiency inhibits the mobilisation of vitamin A stores in rats, and may impair the absorption and utilisation of vitamin A (22). Under normal conditions, the hepatic stellate cells contain over 90% of the liver stores of vitamin A, mainly as retinyl esters. Secretion of retinol from the liver stores to the circulation is thought to be directly from the stellate cells or indirectly via the hepatocytes (23). Interference in this complicated intrahepatic balance between cellular uptake and mobilisation could underlie the effect of iron supplementation on retinol distribution.

Deficiencies of both vitamin A and iron are very prevalent worldwide, and moreover often occur together. However, most efforts to combat these deficiencies are directed towards just one of these micronutrients. In view of the adverse effect of iron supplementation on vitamin A status found in this study, supplementation with iron alone in infants may compromise vitamin A status. This effect would be exacerbated in infants, and perhaps others, with low vitamin A status.

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Iron in the Oceans.

Not unlike the discussion in human nutrition concerning growth-limiting nutrients, is the debate in oceanography about which nutrients limit phytoplankton growth. Many nutrients important for optimal growth in humans, such as iron, nitrogen and phosphorus, are also essential for growth of phytoplankton. Phytoplankton play a crucial role in the conversion of CO_2 to new organic matter. By removing CO_2 from the atmosphere, phytoplankton can influence the process of climate change. It is unlikely that all nutrients are equally limiting at the same time. Residence times of nutrients in the ocean vary considerably, from around 50,000 years for phosphate to only 30 years for iron. Mostly, interest has centred on the fast exchange nutrients such as NO_3^- and Fe. Some groups argue that iron is the limiting factor in at least 30% of the world's oceans. Phytoplankton use iron for both photosynthesis and respiratory electron transfer. The role of iron in oceans is poorly understood however. Dissolved iron is present in very low concentrations (10^{-10} – 10^{-9} moles/kg), mostly bound to ligands. Free iron would precipitate as oxyhydroxides, or be absorbed on sinking particles. Organic ligands like porphyrin and siderophores help to retain iron where it is most needed: near the sea surface where there is sufficient light for photosynthesis. Indeed, phytoplankton biomass can be dramatically increased by addition of Fe or NO_3^-. Recently however, Tyrell argued that these effects are relatively short lived, as the balance between nitrogen fixation and denitrification is ultimately set by influx of phosphate. When more phosphate comes into the oceans, more nitrogen is fixed. The nitrogen cycle is more adaptable than the phosphate cycle and adjusts to the input and loss of phosphate. This is not unlike the concept of type-1 and type-2 nutrients in human nutrition. Thus it is possible to find low concentrations of phosphate in surface sea water, while all available nitrogen has been consumed, even though phosphate is the limiting nutrient.

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CHAPTER 6

Modulation of Interferon-γ, Neopterin and Interleukin-6 by Iron, Zinc and β-Carotene Supplementation.

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Submitted for publication.

ABSTRACT

Micronutrients can have profound effects on cellular and humoral immunity. Micronutrient deficiency is associated with increased risk of morbidity and mortality. Supplementation with various micronutrients has been shown to improve immunocompetence. In the present study, the effects of iron, β-carotene and zinc supplementation on *ex-vivo* cytokine production and macrophage activation were investigated in infants with high prevalence of micronutrient deficiencies.

In a double-blind, placebo controlled intervention trial, 387 infants, aged 4 months, were supplemented for 6 months with iron and/or zinc and/or β-carotene or placebo. Micronutrient status and immune function were assessed at the end of supplementation.

Anemic and iron deficient infants had lower, and zinc deficient infants higher inferon-γ production than non-deficient infants. Plasma neopterin concentrations were lower in iron deficient, and higher in zinc and vitamin A deficient infants. Interferon-γ production was higher in iron, and lower in zinc and β-carotene supplemented infants. Interleukin-6 production exhibited the opposite pattern to interferon-γ production. In concordance with the production of interferon-γ, plasma neopterin concentrations were higher in iron and lower in zinc supplemented infants.

Concluding, iron deficiency depressed, whilst iron supplementation enhanced the type-1 immune response, whereas zinc and β-carotene had opposite immunomodulatory effects to iron supplementation. Thus, micronutrient deficiency as well as supplementation has an important effect on cytokine responses and immune reactivity.

INTRODUCTION

Deficiency of micronutrients can have profound effects on cellular and humoral immunity in humans and experimental animals. Such deficiencies are associated with high morbidity and mortality, especially in infants. Supplementation is commonly used to combat micronutrient deficiencies and to improve health in infants: both vitamin A and zinc supplementation have been shown to reduce morbidity and mortality from infectious diseases (1,2). Iron supplementation is more controversial: positive as well as negative effects have been reported (3,4).

Anemia and iron deficiency are associated with depressed cell-mediated immunity (5). Studies in animals suggest that inadequate supply of iron impairs T-lymphocyte proliferation, whereas humoral immunity is probably less affected. Iron also has a critical role in cytotoxic activity of macrophages as it is required for the production of hydroxyl radicals, although it is unclear to what extent macrophages or other cytotoxic cells are affected by iron deficiency (6). Supplementation with iron has given conflicting results ranging from increased immunocompetence to increased incidence and severity of infections (3).

Zinc deficiency leads to a decrease of both B- and T-lymphocyte numbers, and also reduces antibody response and numbers of cytotoxic cells. Zinc supplementation has been reported to improve the incidence, severity and outcome of infections, especially of diarrheal and respiratory tract infections, but also of malaria (2,4,7).

Severe vitamin A deficiency leads to xerophthalmia and blindness. However, morbidity and mortality are markedly increased in populations with only marginal vitamin A deficiency (8). Decreased cell-mediated immune function, reduced cytotoxic cell activity and reduced antibody titers have all been reported in vitamin A deficiency. However, the exact underlying immune defects are not yet clear (9).Vitamin A supplementation has been shown to reduce infant mortality by about 25% (1). Furthermore, vitamin A supplementation has been shown to reduce the severity of malaria (4).

Status with respect to various micronutrients is a major determinant of optimal host defense. However, not only are the underlying mechanisms by which micronutrients modulate immune function not fully understood, also the exact effects of different micronutrients on immune function are not yet clear. One major problem in the interpretation of host defense defects in micronutrient deficiencies is that often status with respect to more than one micronutrient is suboptimal. Concomitant micronutrient deficiencies can have immunomodulatory effects that counteract or enhance each other, confounding the overall effect.

In this study, the effects of supplementation with iron, zinc and β-carotene on cytokine response and macrophage activation was investigated in infants with a high prevalence of iron, zinc and vitamin A deficiency. We measured *ex-vivo* cytokine production after stimulation in whole blood cultures, as well as plasma neopterin concentrations and micronutrient status.

SUBJECTS AND METHODS

Study design, subjects and procedures.

The study was a randomized, double-blind, placebo-controlled supplementation trial in infants aged 4 months at recruitment. Six groups of infants were supplemented with iron (10 mg/day), zinc (10 mg/day), iron+zinc (10 mg each/day), β-carotene (2.4 mg/day), zinc+β-carotene (10 mg/day and 2.4 mg/day respectively) or placebo. The study was carried out in a rural area of Bogor District, West Java, Indonesia. Infants were supplemented 5 days/week for 6 months by trained village health volunteers.

Mothers were informed of the purpose and procedures of the study. After written informed consent was given by the mother, infants were assessed anthropometrically, and a short history was taken. After 6 months of supplementation, a blood sample was taken of the infant for biochemical assessment of nutritional status and immune function. All infants with a hemoglobin concentration of <110 g/L were given iron supplementation treatment after completion of the trial. The protocol was approved by the ethical committees of the National Health Research and Development Institute of Indonesia and the Royal Netherlands Academy of Arts and Sciences.

Measurements and analysis.

Non-fasting 5 mL venous blood samples (heparinized vacuum tubes from Becton and Dickinson. Leiden, The Netherlands) were taken from the infants. Blood samples were immediately stored at 4°C to prevent microhemolysis and separated within 5 h. Plasma samples were aliquoted and stored at –30 °C until analysis. Cytokine production was measured using an *ex-vivo* whole blood cytokine production after stimulation assay as described by Van Crevel et al (10). Within 15 minutes of collection of the blood sample, 200 l of whole blood, diluted 1:1 with RPMI (RPMI 1640, Dutch Modification, Costar, Badhoevedorp, The Netherlands, with pyruvate, glutamin and garamycine 1% w/v each), was incubated with either phyto-haemagglutinin (PHA-P, Sigma, Zwijndrecht, The Netherlands) in a final concentration of 30 mg/L, lipopolysaccharide (LPS, E. coli serotype 055:b5; Sigma) in a final concentration of 10 µg/L, or PBS as blank control, and then incubated at 37°C for 10 h. After incubation, the supernatant was collected for determination of cytokine concentrations. The concentrations of PHA and LPS used, as well as the incubation time, were chosen to give an optimal response in this system.

Hemoglobin concentrations were measured by a standard cyanoblue method (Humalyzer, Germany). Plasma zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace element-free procedures (11). The CV (10% duplo analysis and pooled control samples) for zinc analyses was <5%. Plasma retinol concentrations were measured by HPLC (MilliporeWaters, Harrow, Middlesex, UK) (12). The CV (10% duplo analysis and pooled control samples) for the retinol and carotenoid analysis was <10%. Ferritin and neopterin concentrations were measured using commercial ELISA kits (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. The cytokines interferon-γ (IFN-γ) and interleukin-6 (IL-6) were also measured with ELISA (Pelikine, CLB, Amsterdam, The Netherlands, calibrated with the standards of the

National Institute of Biological Standards and Controls, Potters Bar, UK) and according to the manufacturer's guidelines. The CV for all ELISA assays was <10%. α1–Acid glycoprotein (AGP) concentrations were measured using immuno-turbidimetric techniques (Cobas Fara analyzer, Roche Products, Welwyn, UK). The CV for the AGP assays was <10%. Plasma AGP concentrations were analyzed to assess the acute phase reaction, which will lower plasma concentrations of zinc and retinol, and raise plasma concentrations of ferritin (13). Also, plasma neopterin concentrations will be affected by infection. Plasma AGP concentrations rise within 24 hours of infection, and stay elevated for up to several weeks after the initial acute phase response (14).

Statistical analysis.

Data were checked for normal distribution using the Kolmogorov-Smirnov test of normality. Plasma concentrations of ferritin, zinc, interleukin-6 and neopterin were transformed to logarithm, and interferon-γ concentrations were transformed to square root before statistical analysis to achieve normality. Differences in cytokine production and plasma neopterin concentrations between the different supplementation groups were tested with analysis of variance (ANOVA). The effect of each supplemented micronutrient on cytokine production and plasma neopterin concentrations was explored with multiple linear regression (MLR), taking sex or AGP concentrations into account as indicated. Differences in cytokine production and neopterin concentration between infants with deficiency of a specific micronutrient and non-deficient infants was tested with the non-parametric Mann-Whitney U test. Statistical analysis was carried out SPSS 7.5.2 (SPSS Inc, Chicago, IL) software package.

RESULTS

Subjects.

Between November 1997 and July 1998, 387 infants from six adjacent villages in rural West Java, Indonesia were enrolled in a supplementation trial with iron, zinc, iron+zinc, β-carotene, zinc+ β-carotene or placebo. After six months of supplementation, at a mean age of 10.2 months [SD ± 0.5], an end-point blood sample was taken to assess biochemical indicators of micronutrient status and immune function. In total 256 infants (66%) completed the trial including an end-point bloodsample. *Ex-vivo* whole blood cytokine production was assessed in 184 of these infants. Results of 13 incubations were excluded from statistical analyses as high blanks indicated possible breach of sterility during incubation, and 8 samples were lost for IFN-γ determination during incubation or analysis. Circulating plasma neopterin concentrations were only measured in the infants who had received iron, zinc, β-carotene or placebo (n=174).

Effect of micronutrient status on *ex-vivo* cytokine production.

The effect of the micronutrient status of the infants, irrespective of supplementation, on *ex-vivo* production of IFN-γ and IL-6 was investigated. As micronutrient status indicators are not reliable in the presence of the acute phase response, all infants with plasma AGP concentrations >1.2 g/L were excluded from the analysis (n=39). IFN-γ production correlated negatively with plasma zinc

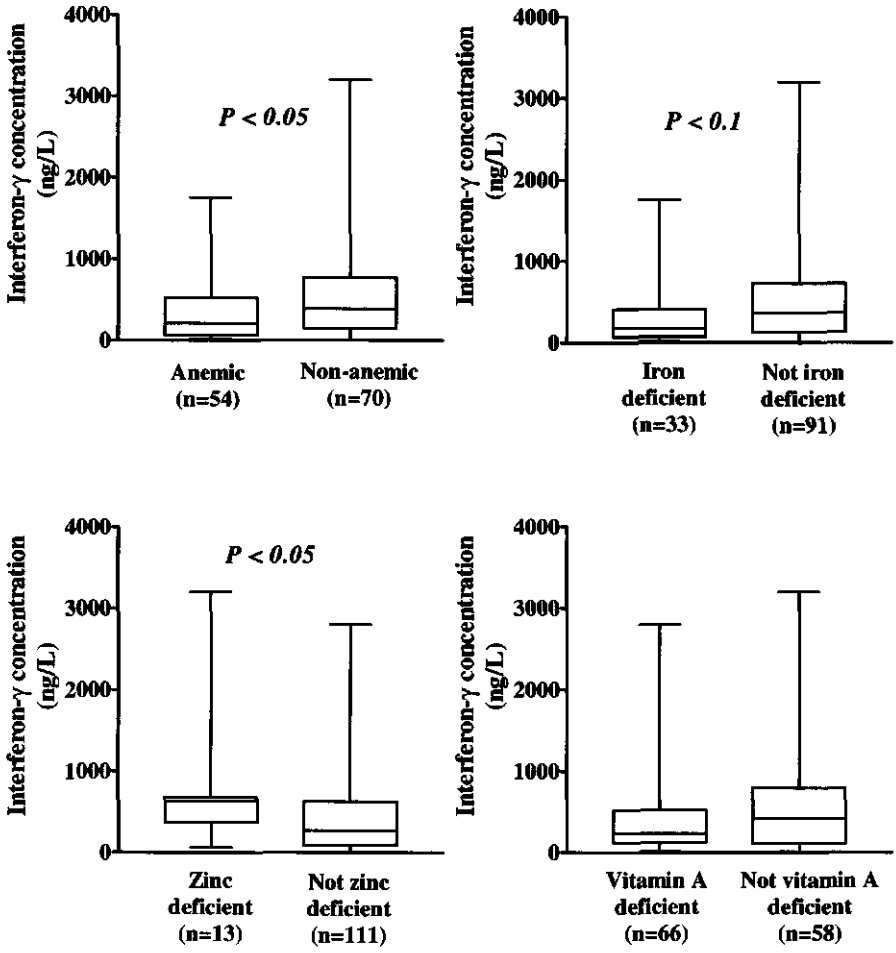

Figure 1. *Ex-vivo* interferon-γ production after stimulation with PHA in infants with anemia or deficiency of iron, vitamin A or zinc, compared to non-anemic or non-deficient infants. Infants (n=39) with an acute phase response (plasma AGP>1.2 g/L) were excluded. Anemia was defined as hemoglobin <110 g/L, iron deficiency as anemia and plasma ferritin <12 μg/L, zinc deficiency as plasma zinc < 10.7 μmol/L, and vitamin A deficiency as plasma retinol < 0.70 μmol/L. Differences between the groups were tested with the Mann-Whitney U test. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data.

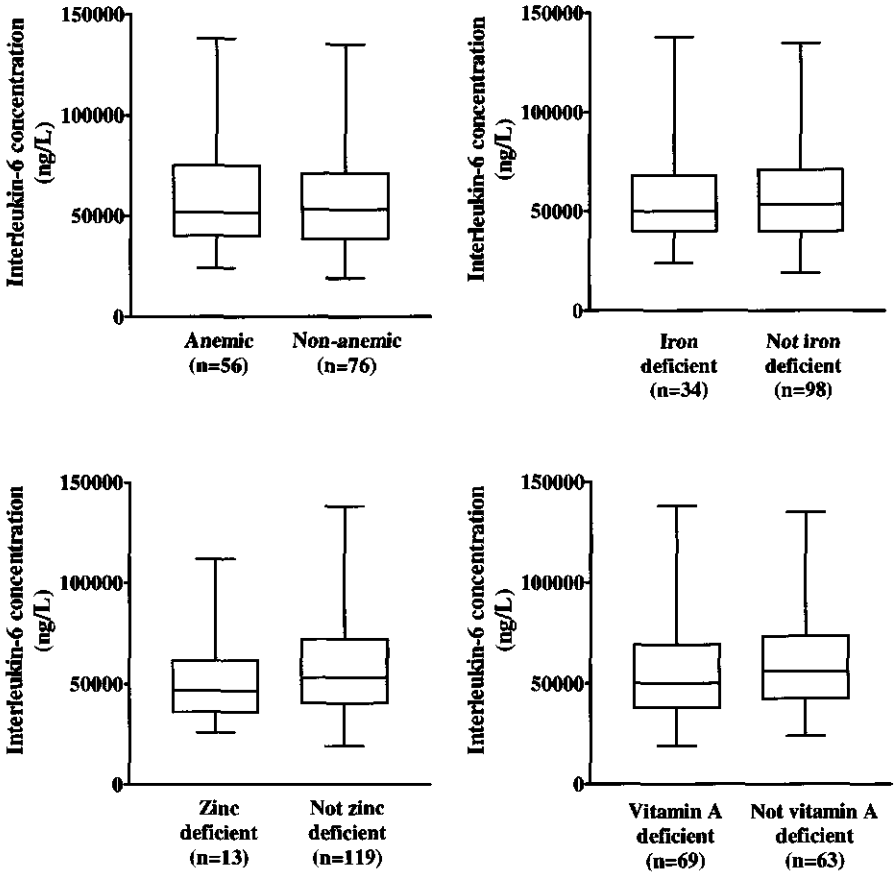

Figure 2. *Ex-vivo* interleukin-6 production after stimulation with LPS in infants with anemia or deficiency of iron, vitamin A or zinc, compared to non-anemic or non-deficient infants. Infants (n=39) with an acute phase response (plasma AGP>1.2 g/L) were excluded. Anemia was defined as hemoglobin <110 g/L, iron deficiency as anemia and plasma ferritin <12 μg/L, zinc deficiency as plasma zinc < 10.7 μmol/L, and vitamin A deficiency as plasma retinol < 0.70 μmol/L. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data.

concentrations (ρ = -0.29, P<0.01, Spearman's rank), but not with other micronutrient status indicators. *Ex-vivo* production of IL-6 weakly correlated with plasma retinol concentrations (r=0.15, P =0.08, Pearson's correlation) and with plasma β-carotene concentrations (ρ =0.21, P<0.05, Spearman's rank). Next, the effect of deficiency of various micronutrients on production of IFN-γ and IL-6 was investigated (**figure 1** and **figure 2**). Anemic infants (hemoglobin concentrations < 110 g/L) had significantly

lower IFN-γ production (P<0.05, Mann Whitney U) than non-anaemic infants. Iron deficient infants (anemia and plasma ferritin concentration < 12 μg/L) also tended towards decreased IFN-γ production but the difference failed to reach significance (P=0.08, Mann Whitney U). In contrast, zinc deficient infants (plasma zinc concentration < 10.7 μmol/L) had higher IFN-γ production (P<0.05, Mann Whitney U) than infants not zinc deficient. The differences in IL-6 production between deficient and non-deficient infants were much smaller, and not significant. Also, cytokine production in vitamin A deficient infants was not different from that in replete infants.

Effect of supplementation on *ex-vivo* cytokine production.

The *ex-vivo* production of IFN-γ was highest in the infants receiving iron, whilst IFN-γ production was lowest in the infants who had received zinc+β–carotene (Kruskal Wallis, P<0.05, **Figure 3**). In contrast, IL-6 production was lowest in the infants who received iron and highest in the infants who had received zinc+β-carotene, however because of the large interindividual variation the difference failed to reach significance (**Figure 4**).

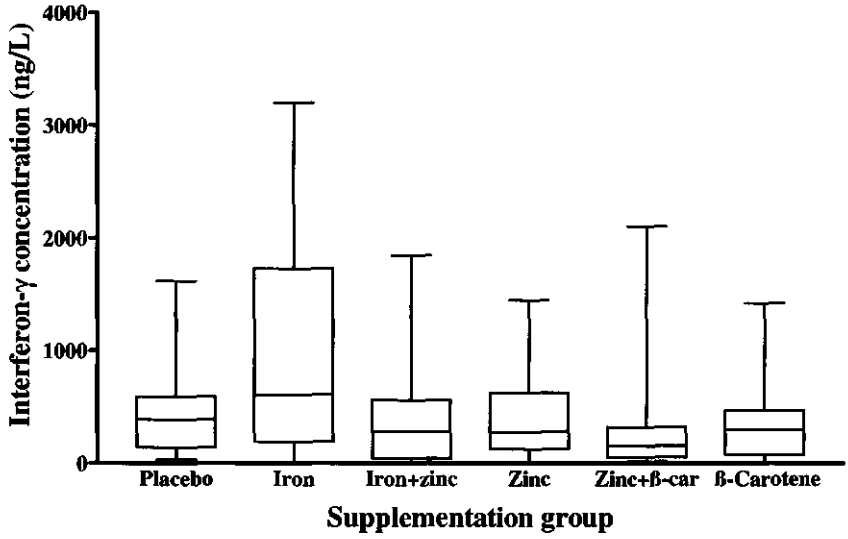

Figure 3. *Ex-vivo* production of interferon-γ after stimulation with PHA in infants supplemented with iron, zinc, and/or β-carotene. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data. Groups are significantly different from each other (P<0.05, Kruskal-Wallis).

The effect of each supplemented micronutrient on *ex-vivo* cytokine production was investigated in a multiple linear regression model. Supplementation with iron was positively related to IFN-γ production (r=0.21, P<0.01), whereas supplementation with

zinc was negatively related to IFN-γ production (r= -0.20, P<0.05). Also β-carotene supplementation was negatively related to IFN-γ production (r = -0.18, P<0.05). However when all 3 supplemented micronutrient were entered into the model simultaneously, β-carotene no longer contributed significantly to the model. *Ex-vivo* IL-6 production was negatively related to iron supplementation (r= -0.14, P=0.07), and postively related to β-carotene supplementation (r= 0.14, P=0.06), but these relationships just failed to reach statistical significance. There was no relationship between IL-6 production and zinc supplementation . Sex and plasma AGP concentrations were initially included as possible confounders, but as they did not contribute to the models, they were not included in the final analyses.

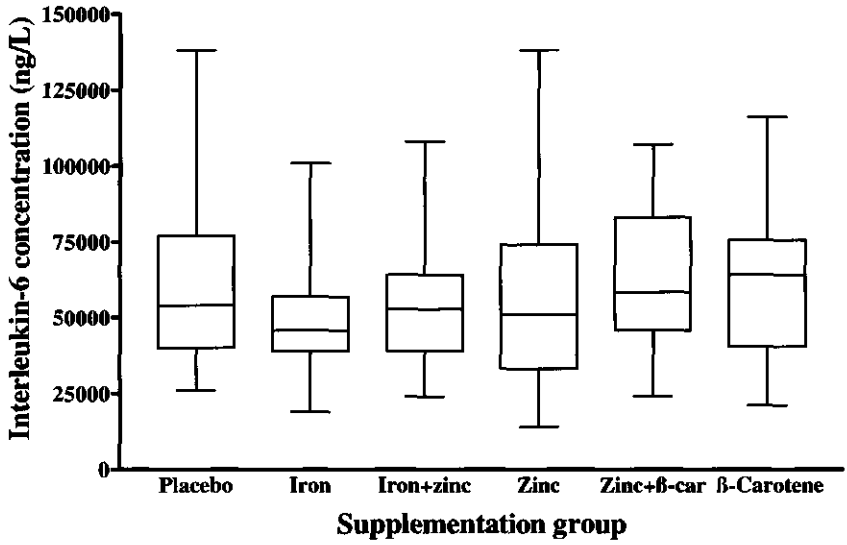

Figure 4. *Ex-vivo* production of interleukin-6 after stimulation with LPS in infants supplemented with iron, zinc, and/or β-carotene. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data.

The effect of micronutrient status on plasma neopterin concentrations.

The relationship between plasma neopterin concentrations and micronutrient status was investigated in a multiple linear regression model, again including plasma AGP concentrations as confounder and irrespectively of supplementation (**Table 1**). Plasma ferritin concentrations were positively related to plasma neopterin concentrations (R=0.21, P<0.01), whereas plasma zinc concentrations were negatively related to plasma neopterin concentrations (R= -0.20, P<0.01). Plasma retinol concentrations were also negatively related to plasma neopterin concentrations (R= -0.16, P<0.05). Hemoglobin concentrations were not significantly related to plasma neopterin concentrations.

Table 1. Relationship between micronutrient status and plasman neopterin concentration.

Micronutrient status indicator	Plasma neopterin concentration	
	R[1]	P
Hemoglobin concentration	0.05	NS
Plasma ferritin concentration	0.21	<0.01
Plasma zinc concentration	- 0.20	<0.01
Plasma retinol concentration	- 0.16	0.04

[1]. Partial correlation coefficient between indicator of micronutrient status and plasma neopterin concentration, taking plasma concentrations of α_1-acid glycoprotein concentrations as indicator for the acute phase response into account as possible confounder.

Micronutrient deficiency was also associated with altered plasma neopterin concentrations (**Figure 5**). Infants (n=39) with a plasma AGP concentration of >1.2 g/L were excluded from statistical analyses to prevent confounding by the acute phase response. Anemic infants had similar plasma neopterin concentrations to non-anemic infants, but infants with iron deficiency anemia had significantly lower plasma neopterin concentrations than infants without iron deficiency (P<0.01, Student's T test). In contrast to iron deficiency, infants with zinc deficiency or with vitamin A deficiency had higher plasma neopterin concentrations than non-deficient infants (P<0.05, Student's T test).

Effect of supplementation on plasma neopterin concentrations.

Circulating plasma neopterin concentrations were measured in the four groups receiving single micronutrient supplements or placebo. Plasma neopterin concentrations were highest in the iron group, and lowest in the zinc supplementation group (**Figure 6**). Iron supplemented infants had significantly higher plasma neopterin concentrations than zinc supplemented infants (P<0.05, ANOVA). The possible confounding effects of the presence of inflammation on circulating plasma neopterin concentrations were taken into account by including plasma AGP concentrations in a multiple linear regression model. Iron supplementation was significantly positively related to plasma neopterin concentrations (R=0.20, P<0.01, MLR controlling for AGP). Zinc supplemention was negatively related to plasma neopterin concentration, but only weakly (R= -0.12, P=0.1, MLR controlling for AGP). β-Carotene supplementation was not significantly related to plasma neopterin concentrations.

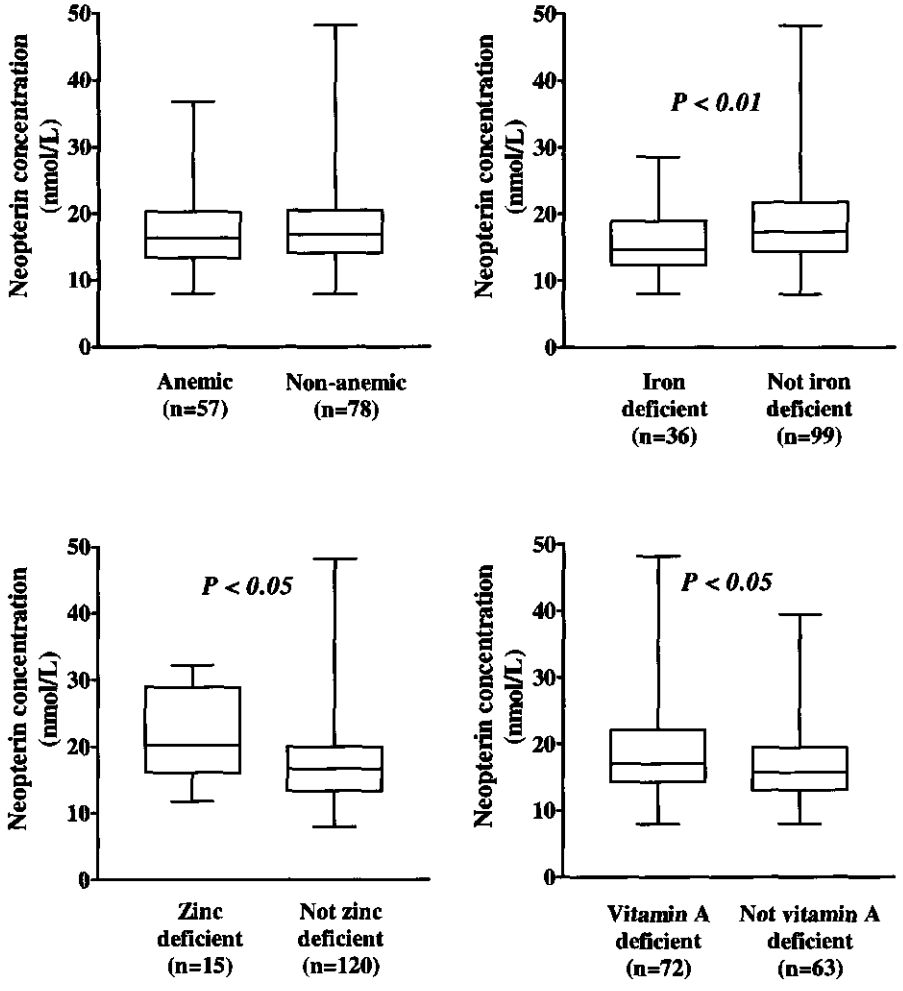

Figure 5. Plasma neopterin concentrations in infants with anemia or deficiency of iron, vitamin A or zinc, compared to non-anemic or non-deficient infants. Infants (n=39) with an acute phase response (plasma AGP>1.2 g/L) were excluded. Anemia was defined as hemoglobin <110 g/L, iron deficiency as anemia and plasma ferritin <12 μg/L, zinc deficiency as plasma zinc < 10.7 μmol/L, and vitamin A deficiency as plasma retinol < 0.70 μmol/L. Differences between the groups were tested with Student's t-test, neopterin concentrations were transformed to logarithms before statistical analyses. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data.

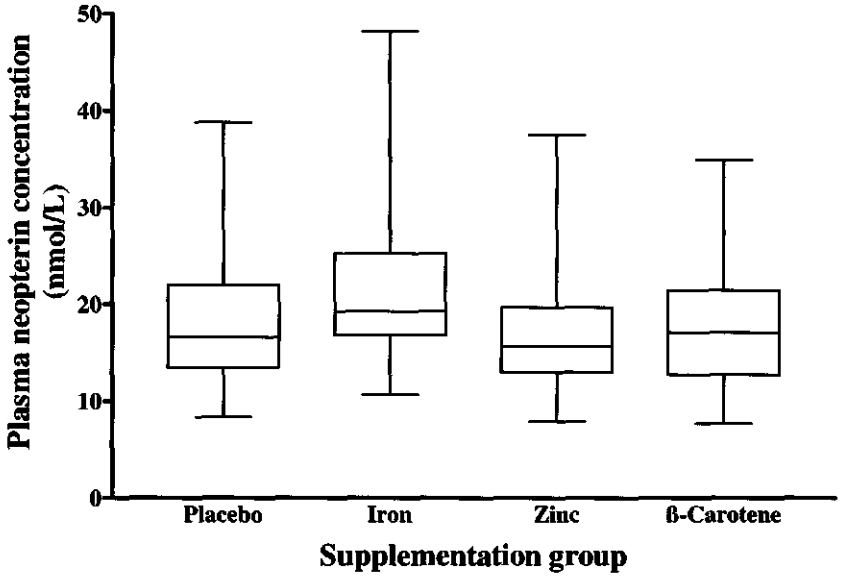

Figure 6. Plasma neopterin concentrations in infants supplemented with iron, zinc, and/or β-carotene. Boxes extend from the 25-th to the 75-th percentile, with the median indicated by the horizontal line. Whiskers show the range of the data. The iron group is significantly higher than the zinc group ($P < 0.05$, ANOVA). Neopterin concentrations were transformed to logarithms before statistical analyses.

DISCUSSION.

This study demonstrates that supplementation of infants with iron increases IFN-γ production while supplementation with zinc or β-carotene reduces IFN-γ production. The effect of supplementation with iron, zinc or β-carotene on plasma neopterin concentrations mirrors the effects on *ex-vivo* IFN-γ production. This is consistent with neopterin being a downstream product of the IFN-γ pathway. Supplementation affected *ex-vivo* production of the type-2 cytokine IL-6 in the opposite way to that of IFN-γ production, with iron supplementation resulting in lower, and β-carotene supplementation in higher IL-6 production.

IFN-γ is important in the initiation of the cellular immune response and the increased production in iron supplemented infants could reflect increased cellular immune reactivity. The effect of iron status in modulating the cellular immune response is further supported by the lower production of IFN-γ in the anemic and iron deficient infants compared to non-anemic or replete infants. Interestingly, IL-6 production was lower in the iron supplemented infants, whereas anemia or iron

deficiency did not affect IL-6 production. IL-6, as a type-2 cytokine, is important for the humoral immune response. The decreased IL-6 production in iron supplemented infants may be due, at least in part, to the increased IFN-γ production in these infants, as IFN-γ downregulates the type-2 cytokine response. This may imply that iron supplementation shifts the cytokine balance towards a type-1 response. At first glance, this would conceptually be contradictory to the known effects of iron supplementation with respect to increasing susceptibility to, and severity of infection. However, part of the explanation could be that the infectious agent also utilises the supplemented iron (15,16). Furthermore, high dose iron supplementation as used in some of the earlier studies could well have different effects on immune function than lower doses of iron such as given in the present study (4). On the other hand, impaired host defense in iron deficiency and improved immunocompetence after iron supplementation have been reported (17-20).

The effects of zinc and β-carotene supplementation on the *ex-vivo* production of IFN-γ and IL-6 were less pronounced than those of iron supplementation, and showed an opposite pattern. Plasma zinc concentrations were negatively correlated to IFN-γ production, and infants with zinc deficiency had higher production of IFN-γ. Thus it would appear that the availability of zinc can dampen type-1 cytokine production. Alternatively, zinc deficiency could upregulate the cell-mediated immune response. These findings are rather unexpected, since the addition of zinc to cell cultures has been found to enhance cytokine production, with a marked increase in IFN-γ production after stimulation with some but not all antigens (21,22). However, results obtained in *in-vitro* experiments may not reflect the conditions of zinc deficiency *in-vivo* or zinc supplementation. In humans, the effect of zinc deficiency or supplementation on immune function is not clear (4,23,24). Results from earlier studies may have been confounded by intercurrent infection which affects both assessment of micronutrient status as well as immune function. Therefore, we excluded subjects with infection as indicated by an acute phase response, from statistical analysis.

The effect of supplementation with micronutrients on cytokine response can be confounded by concurrent deficiency of various micronutrients on immune function. This interrelationship is made even more complex by the fact that micronutrient supplementation can affect the status of not only the micronutrient supplemented but also of other micronutrients. Vice versa, when investigating the effects of micronutrient deficiencies on immune response, the independent effects of micronutrient supplementation on immune function may also play a role. In situations where micronutrient deficiencies are prevalent and supplementation is contemplated however, the same confounding interactions will be present, and will determine the overall effect of supplementation on immune function.

Neopterin is produced by macrophages activated by IFN-γ. Hence, circulating plasma neopterin concentrations can be used as an indicator for *in-vivo* macrophage activity (25). Interestingly, neopterin concentrations were highest in iron supplemented and lowest in zinc supplemented infants, consistent with the effects of supplementation on the *ex-vivo* cytokine production. The high neopterin concentrations in the iron supplemented infants indicates higher activity of the cell-mediated immune system, also resulting in higher IFN-γ production after stimulation. However, higher neopterin

concentrations will also be found during infection. After controlling for infection, neopterin concentrations were still positively related to iron supplementation, and negatively to zinc supplementation.

Furthermore, plasma neopterin concentrations were related to plasma concentrations of ferritin, zinc and retinol, and these relationships were further confirmed by the marked effect of micronutrient deficiencies on plasma neopterin concentrations. Infants with iron deficiency had lower plasma neopterin concentrations, whereas infants with either zinc deficiency or vitamin A deficiency had higher plasma neopterin concentrations. This may reflect higher cell-mediated activity fitting with a type-1 dominance in vitamin A deficiency as described in animal models (26).

Only few studies have investigated neopterin in relation to micronutrients, but a study in South African children did not find an effect of vitamin A status on urinary neopterin concentrations (27). A study in HIV infected adults reported higher serum neopterin concentration in zinc deficiency, similar to the findings in the present study (28). As neopterin production is induced by IFN-γ, these results also support the effects of zinc deficiency on *ex-vivo* IFN-γ production as reported here (29).

The results of this study show that micronutrient supplementation alters cytokine response and immune reactivity. Iron supplementation enhanced, whilst iron deficiency depressed the type-1 immune response. Zinc and β-carotene supplementation had opposite immunomodulatory effects to iron supplementation. However, coexisting deficiencies of other micronutrients can counteract potential benificial effects of supplementation. Supplementation with more than one micronutrient can be expected to be more effective in improving immunocompetence because this strategy will address not only concurrent micronutrient deficiencies, but may also prevent unbalanced immunomodulatory effects of single micronutrients on host defense.

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Indonesian Puppets.

The Indonesian tradition of Wayang puppets is ancient, with references dating as far back as the 9th century AD. Wayang is found on Java and Bali, and probably derives from Indian influences. Wayang performances are always based on the mythical stories from the epic poems Mahabharata and Ramayana. Although the epics have Sanskrit origins they have evolved considerably and are uniquely Indonesian. There are essentially three kinds of Wayang. The shadow theater using elaborately cut-out figures of leather is called Wayang Kulit. Wayang Golek features three-dimensional wooden puppets, exquisitely carved and painted. The least common is Wayang Wong, featuring human dancers. A Wayang performance usually lasts all night, and is accompanied by traditional gamalan music. Although based on the classical epics, personal interpretation, commentary on current affairs and politics, and other themes are all incorporated. For instance in the seventies family planning was propagated through Wayang Kulit performances in the villages. Wayang is a true folk art, and has contemporary value as such. The puppeteer, called dayang, is central to Wayang Kulit and Golek performances, as he composes the story, performs it, chooses the accompanying music, and improvises to keep his audience captivated. He also has a deep knowledge of mythology and Javanese philosophy, and plays an important role in traditional Javanese society as mediator, healer, and adviser. The most intriguing and also most popular Wayang figure is Semar. Superficially a clown, a clumsy servant to the heroes, he is fat, round, and ageless, with a facial expression that is in between laughing and crying. On closer observation however, his comments are wise and cryptic, his role is more of a father than of a servant, and he has an odd impartiality that shakes the very distinction between good and evil. He simultaneously represents the ordinary clumsy human among the mythical heroes of the story, and is a divine and moral presence, commenting on and intervening in the play. Perhaps the ambiguous role of the fool in a Shakespeare play, being simultaneously the lowest character in the story, and the aloof commentator not only on the happenings in the play, but also on the human condition in general, most closely approaches the character of Semar.

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CHAPTER 7

Effects of the Acute Phase Response on Indicators of Micronutrient Status.

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Submitted for publication.

ABSTRACT

Background. The plasma concentrations of many micronutrient status indicators change during infection because of the acute phase response, leading to unreliable measurement of micronutrient status and deficiency.

Objective. In this study, the sensitivity of several micronutrient status indicators to the acute phase response was investigated. Three acute phase proteins, C-reactive protein (CRP), α_1-antichymotrypsin (ACT), and α_1-acid glycoprotein (AGP) were used to quantify the acute phase response and related to plasma concentrations of micronutrient status indicators.

Design. The end-point blood samples of all 418 infants who completed a 6 mo randomized, double-blind, placebo-controlled, supplementation trial with iron, zinc and/or β–carotene were used to analyze relationships between acute phase proteins and micronutrient status indicators in a cross-sectional observational way.

Results. The acute phase response significantly affected indicators of iron, vitamin A and zinc status. Plasma ferritin concentrations were higher, whereas plasma concentrations of retinol and zinc were lower. Hemoglobin concentrations and the MRDR were less affected. Consequently, iron deficiency anemia was underestimated (4.5%), and vitamin A deficiency (4.6%) and zinc deficiency (1.9%) were overestimated in the whole population as compared to infants with no infection. Plasma retinol concentrations were most closely related to plasma concentrations of ACT, whereas plasma concentrations of ferritin and zinc were most closely related to plasma AGP concentrations.

Conclusions. Using micronutrient status indicators without taking the effects of the acute phase response into account will result in a distorted measurement of the micronutrient status, with the extent of the distortion depending on the prevalence of infection in the population.

INTRODUCTION

Micronutrient status defines the amount of a micronutrient available for metabolic functions, with body stores being the most important determinant of micronutrient supply for most micronutrients. Plasma concentrations of micronutrients are often measured as surrogates for total body stores and thus micronutrient status, assuming that the distribution between the different compartments is constant. However during the acute phase response, plasma concentrations of many micronutrients change (1). It is unclear as yet whether these changes solely reflect a redistribution or also changed body stores. Moreover, during infection, micronutrient losses and requirements are higher so eventually body stores will also be affected by the infection. Regardless of the underlying mechanisms, many commonly used indicators of micronutrient status are affected by the acute phase response, and may not adequately reflect micronutrient status during infection.

The extent to which indicators of micronutrient status are affected by the acute phase response depends not only on the specific indicator, but also on the severity of infection, the time phase of the acute phase response, and the nutritional status of the individual (2). Much effort has been put into finding micronutrient status indicators that are not affected by the acute phase response, but with limited success. Another approach however, is to measure the acute phase response and quantify the effects on different micronutrient status indicators, so as to be able to take changes into account. Several studies have looked at vitamin A status in both clinically ill subjects and in apparently healthy subjects in relation to the acute phase response. Plasma retinol concentrations were found to decrease during illness and rise again during recovery, and in healthy children were related to acute phase proteins (3-6). However, the overall effects of the acute phase response on indicators of vitamin A status or other micronutrients are not yet well established enough to be able to predict changes, especially at the population level.

The acute phase response is a generalized reaction of the body to inflammation (7). The proteins most directly involved are referred to as acute phase proteins, but reaction time and magnitude of change after the onset of inflammation, and also their sensitivity to stimuli can differ. C-reactive protein (CRP) is very sensitive, especially to bacterial infections. Plasma concentrations increase within 10 hr of the onset of acute inflammation, and normalize rapidly, usually in a week. α_1-Antichymotrypsin (ACT) is also rises early, but remains elevated for longer than CRP. α_1-Acid glycoprotein (AGP) starts to rise more than 24 hr after the onset of inflammation, but remains elevated well into convalescence, thus can be detected for weeks after the infection, and is also elevated in low grade chronic inflammation (7,8).

In this study the sensitivity of several frequently used indicators of micronutrient status to the acute phase response, and the magnitude of changes through the consecutive stages of infection was investigated. The acute phase response was quantified using the concentrations of three different acute phase proteins. For each indicator of micronutrient status studied, the relationship with each acute phase protein was investigated and the most informative acute phase protein for each indicator of micronutrient status was identified.

SUBJECTS AND METHODS

Study design and location.

Infants were recruited at 4 mo of age for a randomized, double-blind, placebo-controlled supplementation trial with iron (10 mg/day), zinc (10 mg/day), β-carotene (2.4 mg/day), iron plus zinc (10 mg each/day), zinc plus β-carotene (10 mg and 2.4 mg respectively/day) or placebo. Eligible infants were identified by the village health volunteers, and the mothers invited to participate in the study. Mothers were informed of the procedures and purpose of the study, and written informed consent was given by the mother or warden. The study was carried out in a rural area of Bogor District, West Java, Indonesia between October 1997 and March 1999. After 6 mo of supplementation, a blood sample was taken from the infants to assess micronutrient status and acute phase proteins. A blood sample was only collected from infants who were deemed healthy after clinical examination. All infants with a hemoglobin concentration of <110 g/L were given iron supplementation treatment upon completion of the trial. In this study only the relationship between acute phase proteins and indicators of micronutrient status is investigated. The effects of supplementation are reported elsewhere.

A non-fasting 5 mL venous blood sample was taken from the infants. A closed-tube heparinized vacuum system was used to avoid zinc contamination (Becton and Dickinson. Leiden, The Netherlands). Blood samples were immediately stored at 4°C to prevent microhemolysis and separated within 5 h. Plasma samples were aliquoted and stored at –30 °C until analysis.

Five hours prior to the blood sampling, the infants received a small dose of 3,4-didehydroretinol in oil (1.5 mg in 700 µl) for the modified relative dose response (MRDR) test. The MRDR test uses the ratio of 3,4-didehydroretinol to retinol as an indicator of retinol liver stores (9). When retinol liver stores are low, more 3,4-didehydroretinol appears in plasma relative to retinol concentrations. A ratio of > 0.06 is considered indicative of insufficient retinol liver stores (9).

Hemoglobin concentration was measured by the standard cyanoblue method (Humalyzer, Germany). Plasma zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Australia) using trace-element free procedures, as described in an earlier paper (10). The CV (10% duplo analysis and pooled control samples) for zinc analyses was <5%. Plasma retinol and 3,4-didehydroxyretinol concentrations were measured by HPLC (Millipore Waters, Harrow, Middlesex, UK). 3,4-Didehydroxyretinol was measured in the same run as plasma retinol (11). The CV (10% duplo analysis and pooled control samples) for the

retinol analysis was <10%. Ferritin was measured using a commercial ELISA-kit (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. C-reactive protein (CRP), α_1-antichymotrypsin (ACT) and α_1-acid glycoprotein (AGP) were measured using immunoturbidimetric techniques (Dako, Ely, UK) using a Cobas Fara analyzer (Roche Products, Welwyn, UK). The CV for the ferritin, CRP, ACT and AGP assays was <10%.

Ethical approval.

The protocol was approved by the Ethical Committees of the National Health Research and Development Institute of Indonesia and of the Royal Netherlands Academy of Arts and Sciences.

Statistical analysis.

Data was checked for normal distribution using the Kolmogorov-Smirnov test of normality. Plasma concentrations of CRP, ACT, AGP, ferritin and zinc were transformed to logarithms before statistical analysis. Correlations between the plasma concentrations of the acute phase proteins were analyzed using Pearson's correlation, and differences in prevalence were tested with Pearson's chi-square test.

Differences in indicators of micronutrient status between groups of consecutive stages of infection were analysed using analysis of variance (ANOVA). When the overall F test was significant, differences between the groups were further explored with post-hoc multiple comparisons.

The effect of each acute phase protein on each indicator of micronutrient status was further investigated with a multiple linear regression (MLR) model, with the three supplemented micronutrients included as independent factors to correct for possible effects of the supplementation on the relationship between micronutrient status and the acute phase response. The acute phase proteins were analyzed in separate models because of the strong interrelations between them. Sex and age were initially included as possible confounders, but only sex was included in the final analysis of ferritin as in all other cases they did not contribute to the model.

Statistical analysis was carried out with SPSS 7.5.2 (SPSS Inc, Chicago, IL) software package.

RESULTS

Biochemical data on iron and zinc status was available of 418 infants. Acute phase proteins were measured in all 418 infants. Plasma retinol concentrations were measured in 256 of these infants, with a complete MRDR test available for 238 infants. The infants had participated in a supplementation trial in the 6 mo prior to the collection of the blood sample. Of the infants, 90 had received iron supplements, 94 had received zinc supplements, 38 had received β-carotene supplements, 74 had received iron plus zinc supplements, 39 had received zinc plus β-carotene supplements, and 83 infants had received a placebo supplement. The infants had a mean age (± SD) of 10.1 (± 0.6) mo at the time of blood sampling.

TABLE 3
Change of micronutrient status indicators during the consecutive stages of infection.

	Stage of infection[1]			
Concentrations	No infection	Acute infection	Recent infection	Convalescence
Hemoglobin (g/L)[2]	110 (± 11) *(308)*	110 (± 10) *(24)*	109 (± 11) *(37)*	109 (± 12) *(49)*
Plasma ferritin (µg/L)[3]	14.7 (7.7 - 26.1) *(308)*	22.6 (18.2 - 42.1)[5] *(24)*	34.3 (22.9 - 54.0)[6] *(37)*	33.1 (13.2 - 61.4)[6] *(49)*
Plasma retinol (µmol/L)[2]	0.71 (± 0.22) *(194)*	0.59 (± 0.17) *(13)*	0.58 (± 0.24)[5] *(19)*	0.65 (± 0.26) *(30)*
MRDR[4]	0.10 (± 0.05) *(178)*	0.10 (± 0.05) *(13)*	0.08 (± 0.05) *(17)*	0.10 (± 0.07) *(30)*
Zinc (µmol/L)[3]	14.5 (12.4 - 17.0) *(308)*	14.1 (10.4 - 16.7) *(24)*	13.9 (10.5 - 15.3)[5] *(37)*	14.4 (11.6 - 16.1) *(49)*

[1] Stages were defined on basis of raised plasma concentrations of CRP and/or AGP: "No infection", no raised CRP or AGP; "Acute infection", raised CRP, normal AGP; "Recent infection", raised CRP and AGP; "Convalescence", normal CRP, raised AGP.

[2] Mean (± SD), with *(n)* below. [3] Median (IQR) with *(n)* below. [4] MRDR, Modified Relative Dose Response.

Different from "No infection" group: [5] $P < 0.05$ and [6] $P < 0.01$ (ANOVA).

TABLE 4

Perceived prevalence of micronutrient deficiencies, using indicators of micronutrient status sensitive to the acute phase response, for the whole population and for each consecutive stage of infection[1]

	Whole population	Groups according to stage of infection[2]			
Micronutrient deficiency		No infection	Acute infection	Recent infection	Convalescence
Iron deficiency anemia (%)	21.5	26.0	4.2 [4]	2.7 [4]	16.3 [3]
Vitamin A deficiency (%)	58.2	53.6	84.6 [3]	84.2 [3]	60.0
Zinc deficiency (%)	12.9	11.0	29.2 [4]	27.0 [4]	6.1

[1]Micronutrient deficiencies were defined as follows: plasma retinol concentration < 0.70 µmol/L for vitamin A deficiency; hemoglobin concentration < 110 g/L and plasma ferritin concentration < 12.0 µg/L for iron deficiency; plasma zinc concentration < 10.7 µmol/L for zinc deficiency.

[2] Stages were defined on basis of raised plasma concentrations of CRP and/or AGP: "No infection", no raised CRP or AGP; "Acute infection", raised CRP, normal AGP; "Recent infection", raised CRP and AGP; "Convalescence", normal CRP, raised AGP.

Different from "No infection" group: [3] $P < 0.05$ and [4] $P < 0.01$ (chi-square).

TABLE 5

Relationship between indicators of micronutrient status and each acute phase protein using multiple linear regression[1]

	Acute phase protein					
	CRP		ACT		AGP	
Indicator of micronutrient status	β (± SE)	R	β (± SE)	R	β (± SE)	R
Hemoglobin concentration	-0.05 (± 0.07)	-0.05	-0.05 (± 0.38)	-0.05	-0.10 (± 0.32)	-0.12 [2]
Plasma ferritin concentration	0.24 (± 0.02)	0.27 [4]	0.27 (± 0.13)	0.32 [4]	0.31 (± 0.11)	0.35 [4]
Plasma retinol concentration	-0.22 (± 0.02)	-0.23 [4]	-0.30 (± 0.08)	-0.32 [4]	-0.27 (± 0.08)	-0.28 [4]
MRDR	0.14 (± 0.01)	0.14 [2]	0.08 (± 0.02)	0.08	0.05 (± 0.02)	0.05
Plasma zinc concentration	-0.12 (± 0.01)	-0.13 [3]	-0.06 (± 0.04)	-0.07	-0.15 (± 0.04)	-0.15 [3]

[1] CRP, C-reactive protein; ACT, a1-antichymotrypsin; AGP, a1-acid glycoprotein; MRDR, modified relative dose response. Plasma concentrations of CRP, ACT, AGP, ferritin and zinc were transformed to logarithms before analysis. In the multiple linear regression, the supplemented micronutrients the infants received prior to the blood sampling were included to control for possible confoundig effects. Sex was also included as confounder in the analysis of ferritin.

[2] $P < 0.05$; [3] $P < 0.01$; [4] $P < 0.001$

The effects of the acute phase response on indicators of micronutrient status were further investigated using multiple linear regression (**Table 5**). The relationship of each acute phase protein with each indicator of micronutrient status was analyzed. The supplemented micronutrients the infants received prior to the blood sampling were included in the model as independent factors, to correct for possible effects of supplementation on the relationship between micronutrient status and the acute phase response. Sex was included in the analysis of plasma ferritin concentrations as confounder.

Plasma ferritin concentrations were significantly positively related to all three acute phase proteins, but most strongly to plasma concentrations of AGP (R= 0.27 for CRP; R=0.32 for ACT; R=0.35 for AGP, $P<0.001$ for all). Plasma retinol concentrations were significantly negatively related to plasma concentrations of CRP (R= -0.23, $p<0.001$), AGP (R=-0.28, $P<0.001$) and most closely to plasma concentrations of ACT (R= -0.32, $P<0.001$). Plasma zinc concentrations were significantly negatively related to plasma CRP concentrations (R= -0.13, $P<0.01$) and plasma AGP concentrations (R= -0.15, $P<0.01$), but not to plasma ACT concentrations. The MRDR and hemoglobin concentrations were related to only a single acute phase protein. Hemoglobin concentrations were related to plasma concentrations of AGP (R= -0.12, $P<0.05$), perhaps reflecting a depression of erythropoiesis in later stages of infection. The MRDR was related to plasma CRP concentrations (R= -0.14, $P<0.05$), which might be related to the decreased mobilisation of retinol during the acute phase response.

DISCUSSION

This study shows that the acute phase response distorts the measurement of micronutrient status, by affecting the plasma concentration of commonly used indicators. These effects can in part be considered transient, and directly related to the acute phase response, as the consecutive phases of infection affect the plasma concentrations of the indicators differently. However, the effects of the acute phase response continue well into the convalescence phase of infection. These findings have important consequences for the estimation of micronutrient deficiencies in populations. In the present study, the perceived prevalences of vitamin A deficiency and zinc deficiency were significantly higher, whereas the perceived prevalence of iron deficiency anemia was significantly lower in the presence of an acute phase reaction.

Some frequently used indicators of micronutrient status, such as plasma concentrations of ferritin, retinol and zinc are definitely sensitive to the acute phase response. Other indicators such as hemoglobin concentrations and the MRDR are much less affected by the acute phase response. As these indicators represent nutrient balance over a longer time period, changes in for instance infection state are not immediately reflected. At the population level, the effect of the acute phase response on sensitive indicators of micronutrient status is significant but small. During infection, micronutrient indicators are not related to micronutrient status in the same way as in the absence of infection. Hence, including a measure of the acute phase response in the determination of micronutrient status will enable either correction for the effects of the acute phase response, or exclusion of subjects with an infection. This is especially

important when comparing populations, as the overall effect of the acute phase response on the measurement of micronutrient status is dependent on the prevalence of infection in the population. Infection patterns and prevalence can vary enormously between populations and subgroups. Furthermore, several studies have shown that clinical examination does not rule out the presence of an acute phase response (6,10). Hence, the micronutrient status of populations cannot be confidently compared without taking the acute phase response into account.

The effect of the acute phase response on the plasma concentrations of micronutrient status indicators can be the result of two different mechanisms. During an infection, the requirements and/or losses of a micronutrient are increased, sometimes in combination with impaired absorption, resulting in a real change in body stores (13). Alternatively there can be a redistribution of the indicator of micronutrient status, without a real change in the total body content of the micronutrient, resulting in a distorted measurement of the micronutrient status (14).

To clarify the relative importance of both mechanisms, the pattern of change in plasma concentrations throughout the consecutive phases of the acute phase response can be examined. CRP rises within 10 hr of an infection, whereas AGP only after 24 hr. The most substantial decrease in plasma concentrations of retinol and zinc occurs during the acute infection phase, before the AGP concentrations have risen. During the convalescence phase the plasma concentrations rise again. Plasma zinc concentrations recover almost to the no-infection level, while plasma retinol concentrations rise also, but not quite to the no-infection level. The rapid decrease and the subsequent (partial) recovery of plasma concentrations during the consecutive phases of the acute phase response implies that the first and most substantial effect of the acute phase response is the result of redistribution, rather than changes in body stores. The remaining deficit in plasma retinol concentrations during the convalescence phase may reflect the real change in body stores incurred during the infection.

The importance of the redistribution effect is further supported when the indicators that appear to be less sensitive for the acute phase response are considered. Hemoglobin concentrations and anemia prevalence are hardly affected by infection. Also, the MRDR does not really change during the consecutive phase of infection indicating that retinol liver stores, and thus vitamin A status, is hardly affected, despite the substantial changes in plasma retinol concentrations. Changes in plasma ferritin concentrations during the acute phase response are different from the changes of plasma retinol and zinc concentrations as ferritin itself can be considered an acute phase reactant. In view of this double role, plasma ferritin concentrations cannot reliably reflect iron status unless the presence of infection is excluded.

Are low plasma concentrations of micronutrients during infection a sign of deficiency, and is there an increased risk of clinical symptoms of deficiency? When the low plasma concentrations are caused by a redistribution, they do not necessarily reflect lower tissue concentrations (15). For instance, there is evidence of increased concentrations of zinc and iron in specific tissues such as the liver and the reticulo-endothelial system. Also, the lower plasma concentrations during the acute phase response will rebound when infection has resolved, at least partially. As to yet, it is

unclear whether the lowering of plasma concentrations of micronutrients carries a physiological benefit, except for iron where the redistribution has clear advantages. However, on the other hand there is evidence that the lower plasma retinol concentrations during infection can imperil the retinol supply to the eye, and induce acute clinical ocular signs of deficiency (16). Also, in some instances in may well be that supplementation during infection can have beneficial effects even when there is no deficiency. Such is the case for measles infection and vitamin A supplementation (17).

Furthermore, this study shows that the effect of the acute phase response varies among the different indicators of micronutrient status and with the stage of infection. Each acute phase protein rises at a different stage of the acute phase response. To be able to quantify the effects of the acute phase response on the different indicators of micronutrient status, it is important to know which acute phase protein is the most informative for each indicator of micronutrient status. In this study, plasma retinol concentrations were most strongly correlated with plasma ACT concentrations, whereas plasma zinc and ferritin concentrations were most strongly correlated with plasma AGP concentrations. Although plasma CRP concentrations appear to be the least correlated of the three acute phase proteins with the indicators of micronutrient status, CRP might in practise be more useful than ACT as the cut-off values for CRP are more established than for ACT. Also, ACT used with the present cut-off value of 0.6 g/L identified only a small number of subjects as having an acute phase response, and thus might lack sensitivity. The fact that AGP is strongly correlated to indicators of micronutrient status shows that the effects of infection on these indicators continue well into the convalescence phase, which might be weeks after the initial infection.

The results of this study show that the acute phase response significantly affects the plasma concentrations of commonly used indicators of micronutrient status. Prevalences of vitamin A deficiency and zinc deficiency can be overestimated, whereas the prevalence of iron deficiency anemia can be underestimated if the effect of the acute phase response on the plasma concentration of indicators of micronutrient status is not taken into account. The different indicators of micronutrient status are not affected in the same way or to the same extent through different stages of infection, and thus different acute phase proteins are required to optimally quantify the effect of the acute phase response on specific indicators of micronutrient status. Based on the results of this study, AGP is the most useful indicator of the acute phase response when determining zinc and iron status, whereas ACT is most informative concerning vitamin A status. At population level, using micronutrient status indicators without taking the effects of the acute phase response into account, will result in a distorted measurement of the micronutrient status, with the extent of the distortion depending on the prevalence of infection in that population.

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King of the Sea.

The coelacanths (Latimera chalumnae) are the only surviving species of a prehistoric lineage of fishes, known only from fossil finds until the discovery of a live specimen off the coast of South Africa in 1938. The coelacanth is not just a "living fossil", but the species is related to the ancestors of the land-living vertebrates, and its unique anatomical structures, and physiological and neurological functioning could be precursory to the development of terrestrial life. The coelacanth was thought to have quietly survived as an isolated population of around 500 fish near the Comoro Islands, an evolutionary remnant on its way to extinction. They have gone unnoticed for so long because they are deep-sea fish, living in caves at around 180 meters depth. In 1998 Erdmann et al report the existence of a living population of coelacanths off the northeast coast of Sulawesi. In fact it was Mr Erdmann's wife who first saw a coelacanth on a fish-market in Manado a year earlier, but could not persuade the buyer of the fish to part with it. Interviews revealed that fishermen know the fish and even have a specific name for it (Raja Laut: king of the sea). It is amazing that a huge fish (coelacanths are up to 1.6 meters in length) can be newly discovered, in an era where every square centimeter of the earth has been mapped. Concern is warranted however, as population pressure and industrialisation are rapidly destroying the environment, also in Indonesia, and the little known, incompletely explored world which lies underwater is extremely vulnerable. The ecosystems of reefs and seas are complex and easily damaged. In this context it is sad to note that the coelacanth's conservation status is "insufficiently known to justify conservation action". Some efforts at marine conservation and increasing awareness of the marine environment are being made however. The Wallacea project is describing the flora and fauna of the marine parks in Indonesia systematically, and the Aseanarean initiative is linking the marine ecosystems of the whole South-east Asian region, and the regulations and conservation efforts affecting them. However more research, funds, time and effort are definitely needed!

Erdmann MV, Caldwell RL, KasimMoosa M. Indonesian 'King of the Sea' discovered. Nature. 1998;395:335. Forey P. A Home from Home for Coelacanths. Nature. 1998;395:319.

CHAPTER 8

Supplementing Indonesian Pregnant Women with β-Carotene and Zinc in Addition to Iron and Folic Acid Affects Birth Weight and Incidence of Pregnancy Complications.

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Submitted for publication.

ABSTRACT

Background. Micronutrient deficiencies are prevalent in pregnant women, and adversely affect pregnancy outcome, including maternal and neonatal complications and birth weight. Pregnant women are often supplemented with iron and folic acid, but deficiencies of other micronutrients such as zinc and vitamin A are prevalent also.

Objective. To investigate whether supplementing pregnant women with β-carotene and zinc in addition to iron and folic acid can reduce pregnancy complication rate and increase birth weight.

Design. In a randomized double-blind trial, 229 women 10-20 weeks pregnant, were supplemented until delivery with iron and folic acid, in combination with zinc and/or β-carotene or neither. Maternal and neonatal complications and birth weight were monitored as well as micronutrient status during pregnancy

Results. Supplementation of pregnant women with β-carotene and zinc in addition to iron and folic acid increased birth weight in boys. However, women supplemented with only zinc added to iron and folic acid had more complications during delivery. Micronutrient status indicators during pregnancy were significantly affected by progressive hemodilution, but not discernably by supplementation.

Conclusions. As pregnant women are likely to be at risk of more than one micronutrient deficiency, supplementation of more micronutrients than only iron and folic acid during pregnancy can not only improve pregnancy outcome, but also enhance effectiveness of supplementation and prevent possible antagonistic interactions between micronutrients. Only supplementation of pregnant women with the combination of β-carotene and zinc but not with either zinc or β-carotene alone, in addition to iron and folic acid, improved pregnancy outcome in this study.

INTRODUCTION

Pregnant women are very vulnerable with respect to micronutrient deficiencies, because of increased requirements and grave consequences of deficiency during pregnancy. Micronutrient deficiencies in pregnant women are associated with increased maternal mortality, increased incidence of complications during pregnancy and labor, lower birth weight, and increased incidence of neonatal complications (1). Iron deficiency is the most prevalent micronutrient deficiency, affecting over 35% of the women worldwide (2). Supplementation of pregnant women with iron and folic acid is standard practice in many countries. However, a number of other micronutrient deficiencies are also prevalent, especially in developing countries.

There are indications that zinc deficiency may be common in pregnant women, especially when they are iron deficiency, as the same dietary risk factors are involved. Zinc deficiency has been implicated in pregnancy complications, and supplementation has been shown to improve birth weight (3,4). An additional reason for concern is that iron and folic acid supplementation reduces zinc absorption, and can impair zinc status (5-7). Although clear evidence is lacking, zinc is thought to be implicated in vitamin A metabolism, and zinc deficient animals have lower plasma retinol concentrations (8).

Vitamin A is another micronutrient which is commonly deficient in many developing countries, and associated with increased morbidity and mortality. Furthermore, vitamin A has been shown to have synergistic effects on iron metabolism, and supplementation of pregnant women with vitamin A in addition to iron reduced the prevalence of anemia more than did iron supplementation alone (9). Supplementation with vitamin A and also with β-carotene reduced maternal mortality in Nepal, although the underlying mechanisms are not completely clear (10). However, vitamin A also has teratogenic effects especially during the first trimester, and only low doses of vitamin A (<10,000 IU per day or <25,000 IU per week) are considered safe during pregnancy. β-Carotene is a precursor to retinol, and is considered safe during pregnancy (11).

In Indonesia, maternal mortality is still high, 65 per 10,000 live births (12), despite major improvements in health care quality and access. Poor health and nutritional status of pregnant women are major risk factors, also leading to a high prevalence of low birth weight. Low birth weights are directly associated with high neonatal morbidity and mortality, and the detrimental effects continue well into infancy.

The aim of this study was to investigate whether supplementing pregnant women with β-carotene and zinc in addition to iron and folic acid can reduce the incidence of complications during pregnancy and delivery. In addition, birth weight and health of the neonate was also examined.

SUBJECTS AND METHODS

Study design and location.

The study was designed as a four cell, randomized, controlled, double-blind supplementation trial. The control group received standard supplementation with iron and folic acid. Pregnant women were recruited from 13 adjacent villages in a rural area in Bogor District, West Java, Indonesia. Women were assigned to one of the four groups on basis of individual randomization. All women received iron and folic acid (30 mg/day and 0.4 mg/day respectively) supplementation. In addition, one group received β-carotene (4.5 mg/day), one group zinc (30 mg/day), and one group β-carotene+zinc (4.5 mg/day and 30 mg/day respectively). The iron was given as ferrous fumerate, the zinc as zinc sulphate and the β-carotene as a granulate preparation. Supplements were prepared by the pharmacy of the Gelderse Vallei Hospital (Bennekom, The Netherlands), given as capsules, and taken daily until delivery.

Subjects and procedures.

Pregnant women with a gestational age of <20 weeks were identified by village health volunteers or by local midwifes and invited to participate in the study. Women were informed of the procedures and purpose of the study. After written informed consent was given, pregnancy duration was estimated using a detailed amenorrhea history and physical examination, and an expected date of delivery was calculated. Women were anthropometrically assessed, a history concerning socio-economic status, previous pregnancies, dietary habits, and health was taken, and a 3 mL blood sample was collected. Exclusion before recruitment was on grounds of a gestational age of <10 or >20 weeks, chronic disease, or previous pregnancies with complications.

At recruitment, every subject received a personal bottle, labeled with the subject's name, subject number, and date, containing 40 capsules. Bottles were replaced every month with a new bottle containing 40 capsules,. The number of capsules remaining each month were counted to estimate the number of capsules taken by the subject as a measure of compliance.

At the monthly follow-up during pregnancy, the women were anthropometrically assessed, and a short history concerning health and diet was taken. Minor complaints were addressed. When women had a gestational age of approximately 8 mo, a 3 mL blood sample was collected for biochemical assessment of micronutrient status, and women were screened for hypertension, albuminurea and anemia. Minor complaints were addressed, women with severe anemia (hemoglobin <80 g/L) were advised to deliver in the local health center, and major complaints or symptoms were referred to appropriate medical care. All women received a card to record the details of delivery, the newborn and the birth weight. The cards used pictograms to score delivery complications and neonatal condition. Cards had to be filled in and signed by the birth attendant, midwife, or local health volunteer, preferably on the day of delivery, but not later than 3 days after birth. To facilitate accurate weighing of the newborn, verified scales were supplied, and the health volunteers and midwifes were trained.

Methods.

Anthropometry included measurement of weight, height and mid-upper-arm circumference by trained anthropometrists using standard methods. Non-fasting, morning blood samples were collected using a closed-tube heparanized vacuum system to avoid zinc contamination (Becton and Dickinson. Leiden, The Netherlands). Blood samples were immediately stored at 4°C to prevent microhemolysis and separated within 5 h. Plasma samples were aliquoted and stored at -30°C until analysis.

Hemoglobin concentrations were measured by the standard cyanoblue method (Humalyzer, Germany). Plasma zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace-element free procedures, as described in an earlier paper (13). The CV (10% duplo analysis and pooled control samples) for zinc analyses was <5%. Plasma retinol and β-carotene concentrations were measured by HPLC, in two separate runs as described by Ehrhardt et al.(14). The CV for retinol and carotenoid analyses (10% duplo analysis and pooled control samples) was <5%. Ferritin was measured using a commercial ELISA-kit (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. C-reactive protein (CRP) and α1-acid glycoprotein (AGP) were measured using immunoturbidimetric techniques at the Northern Ireland Centre for Diet and Health, University of Ulster, Northern Ireland (Cobas Fara analyzer, Roche Products, Welwyn, UK). The CV for the ferritin, CRP and AGP assays was <10%.

Ethical approval.

The protocol was approved by the ethical committees of the National Health Research and Development Institute of Indonesia and the Royal Netherlands Academy of Arts and Sciences.

Statistical analysis.

Data was checked for normal distribution using the Kolmogorov-Smirnov test of normality. Differences in prevalence were tested with Pearson's chi-square test, or when numbers were small (e.g. delivery complications) with Fischer's exact test. Differences among groups were tested with analysis of variance (ANOVA), or Kruskal Wallis for non-parametric data. Differences in biochemical indicators among supplementation groups were analysed using ANOVA, and differences in birth weight with analysis of covariance (ANCOVA), with sex as covariate. Plasma concentrations of ferritin and β-carotene were transformed to logarithms before statistical analysis. Differences between recruitment and 8 mo of pregnancy were tested per group using paired T-test, or Wilcoxon rank test for non-parametric data. Differences among the supplementation groups in changes in biochemical indicators between recruitment and 8 mo of pregnancy were analysed with repeated measurements multivariate ANOVA statistics. When the overall F-test was significant, differences among the groups were further investigated with post-hoc multiple comparisons for ANOVA. Statistical analysis was carried out with SPSS 7.5.2 (SPSS Inc, Chicago, IL) software package.

RESULTS

Between July 1998 and January 1999, 229 pregnant women with a gestational age between 10 and 20 weeks were recruited from 13 adjacent villages in Bogor District, Indonesia. A blood sample was collected at recruitment (all women) and of 164 women at approximately 8 mo of pregnancy (**Figure 1**). Information on delivery and birth outcome was obtained from 179 pregnancies. Twenty-two of these women missed the 8 mo blood sample collection. Forty-three women dropped out before, and 7 women after, the collection of the 8 mo blood sample for various reasons (**Figure 1**). Spontaneous abortions (n= 7) occurred in all supplementation groups.

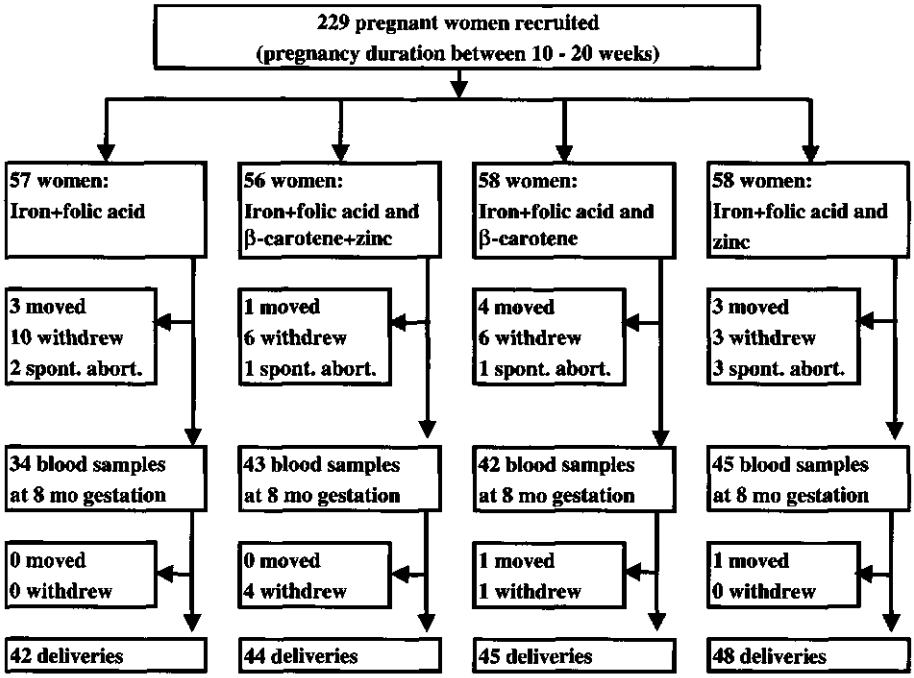

Figure 1. Trial Profile

The age of the pregnant women at recruitment ranged from 15 to 40 y, with a median parity of 2 (**Table 1**). Thirty-two percent of the women were primigravida. As the women were already pregnant when height and weight were measured at recruitment, the midupper arm circumference is preferred as indicator for anthroprometrical status. Mean values were just above the cut-off for undernutrition (<23.3 cm) as used in Indonesia. Hemoglobin concentrations did not differ among the groups at recruitment, and mean values were below the cut-off for anemia (< 120 g/L). However, hemodilution already starts early in pregnancy so hemoglobin concentrations at recruitment may already have been subject to dilution effects, making interpretation more difficult.

TABLE 1

General characteristics of the pregnant women at recruitment.[1]

	Supplementation groups			
	Control	β-Carotene +zinc	β-Carotene	Zinc
n	57	56	58	58
Age (yr)[2]	23.8 (± 5.6)	25.5 (± 5.2)	26.2 (± 6.2)	24.9 (± 5.3)
range	*15 - 40*	*16 - 40*	*15 - 40*	*17 - 37*
Parity[2]	2 (1-3)	2 (1-4)	2 (1-4)	2 (1-4)
range	*1 - 7*	*1 - 8*	*1 - 12*	*1 - 11*
Primigravida (%)	40	36	28	32
MUAC (cm)[2]	23.6 (± 2.1)	24.1 (± 2.3)	24.2 (± 2.3)	24.2 (± 2.3)
Hemoglobin (g/L)[2]	117 (± 15)	115 (± 12)	117 (± 12)	115 (± 10)

[1] No differences between the groups for any of the baseline characteristics.

[2] Mean (± SD) or median (IQR)

When the women were approximately 8 mo pregnant, after 4 mo or more of supplementation, micronutrient status was assessed (**Table 2**). As a result of progressive hemodilution and other physiological changes during pregnancy, concentrations of hemoglobin, plasma retinol and plasma zinc decreased significantly between recruitment and 8 mo of pregnancy in all groups ($P < 0.01$, paired T-test). Plasma concentrations of ferritin decreased in all groups, but to a lesser extent in the β-carotene+zinc group ($P = 0.055$ for the β-carotene+zinc group, $P < 0.01$ all other groups, paired T-test). At 8 mo of pregnancy, there was no difference among the groups in the plasma concentrations of retinol, ferritin, zinc and the hemoglobin concentration (ANOVA). However, at 8 mo of pregnancy plasma concentrations of β-carotene were higher in the women receiving either β-carotene or β-carotene and zinc ($P < 0.05$, ANOVA). The decrease in plasma β-carotene concentrations between recruitment and 8 mo of pregnancy was only significant in the zinc group ($P < 0.01$, Wilcoxon signed rank test).

TABLE 2

Indicators of the micronutrient status of the pregnant women at recruitment and at 8 mo of pregnancy.

	Supplementation groups			
	Control	β-Carotene +zinc	β-Carotene	Zinc
n	34	43	42	45
Concentrations[1]				
Hemoglobin (g/L)				
Recruitment	118 (± 14)	116 (± 12)	118 (± 12)	114 (± 11)
8 months pregnant	109 (± 12)[2]	109 (± 11)[2]	107 (± 12)[2]	106 (± 14)[2]
Plasma ferritin (μg/L)[1]				
Recruitment	24 (8.7-39.6)	21 (10.1-43.2)	25.0 (16.4-39.6)	28 (14.2-46.7)
8 months pregnant	14 (7.8-22.8)[3]	16 (8.8-25.3)[4]	16.2 (8.5-24.4)[3]	14 (7.0-22.3)[3]
Plasma retinol (μmol/L)				
Recruitment	1.1 (± 0.26)	1 (± 0.27)	1 (± 0.20)	1 (± 0.25)
8 months pregnant	0.8 (± 0.29)[2]	0.8 (± 0.31)[2]	0.8 (± 0.31)[2]	0.9 (± 0.24)[2]
Plasma β-carotene (μmol/L)[1]				
Recruitment	0.2 (0.13-0.30)	0.3 (0.15-0.38)	0.21 (0.15-0.29)	0.3 (0.16-0.34)
8 months pregnant	0.2 (0.10-0.23)	0.2 (0.14-0.32)[5]	0.22 (0.14-0.34)[5]	0.2 (0.08-0.29)[3]
Plasma zinc (μmol/L)				
Recruitment	11 (± 3.5)	11 (± 4.3)	12 (± 3.9)	12 (± 4.1)
8 months pregnant	9.1 (± 3.0)[2]	9.2 (± 4.1)[2]	8.2 (± 3.1)[2]	9.1 (± 2.9)[2]

[1] Mean (± SD) or median (IQR); Ferritin and β-carotene concentrations were transformed to logarithm before statistical analysis when necessary.

[2] Significant decrease from recruitment (P<0.01, Paired T test).

[3] Significant decrease from recruitment (P<0.01, Wilcoxon rank test).

[4] Decrease from recruitment (P=0.055, Wilcoxon rank test).

[5] Significantly higher than plasma β-carotene concentration at 8 mo in the zinc group (P<0.05, ANOVA)

TABLE 3

Pregnancy outcome and delivery complications per supplementation group.

	Supplementation groups			
	Control	β-Carotene +zinc	β-Carotene	Zinc
n (deliveries)	42	44	45	48
Sex ratio (M:F)	27:16	29:16	23:23	27:21
Delivery statistics				
Primigravida (n)	14	15	9	12
Preterm (n)[1]	4	2	2	3
Stillborn / neonatal death (n)	0	1	1	4
Deliveries with complications	3	4	5	12[2]
Prolonged labor	1	1	1	4[3]
Retention of placenta	0	2	1	5[3]
Postpartum hemorrhage	2	2	3	1
Meconium in amniotic fluid	0	0	1	2
Breech presentation	0	1	1	3
Cesarean section	0	0	1	1
Puerperal fever	0	0	0	1
Deliveries with neonatal complications	6	6	6	12
Preterm delivery	4	2	2	3
Twins	1	1	1	0
Blue / floppy (neonatal hypoxia)	1	0	0	6[4]
Neonatal jaundice	1	2	2	1
Fever / not drinking	1	1	1	0
Umbilical infection	0	1	0	1
Congenital abnormalities	1	0	1	2
Stillborn / neonatal death	0	1	1	4

[1] Definitions of complications. Preterm delivery: hosptial diagnosis or birth weight < 2.5 kg with pregnancy duration < 37 weeks. Prolonged labor: >12 h. Retention of placenta: >30 min. Postpartum hemorrhage: blood loss >1 L.

[2] $P < 0.05$ (chi-square).

[3] $P < 0.05$ (Fisher's exact test, prolonged labor and retention of placenta were combined).

[4] $P < 0.05$ (Fisher's exact test).

In total, information on 179 deliveries was available (**Table 3**). Eleven women delivered preterm (defined as a hospital diagnosis of preterm delivery or a delivery before 37 weeks of estimated gestational age combined with a birth weight below 2.5 kg). All women (3) with twin pregnancies delivered preterm. Six pregnancies ended in stillbirth or neonatal death. One full term infant was stillborn, with no certain cause diagnosed. One preterm neonate died within one day of birth. Three neonates had congenital abnormalities incompatible with life, and one normal, term neonate died within 7 days of birth with suspected umbilical infection. One full term infant was born with a pes equinus of the left foot, but was otherwise healthy. Birth weight, and complications of the delivery and the neonate were recorded preferably on the day of birth, but always within 3 days, by the birth attendant, midwife, or health volunteer. Deliveries with complications were significantly more frequent in the zinc group compared to all other groups (P <0.05, chi-square). The frequency of most specific complications was not high enough to allow meaningful statistical testing, except for the combination of prolonged labour and/or retentio placenta, both indicative of uterine contractile dysfunction, which was significantly higher in the zinc group as compared to all other groups (P <0.05, Fischer exact test). Although the overall frequency of neonatal complications was not significantly different among the groups, there is a disturbingly higher frequency of neonatal complications in the zinc group. Dividing the complications into specific categories shows that there were significantly more neonates with signs of hypoxia in the zinc supplementation group as compared to the β-carotene or β-carotene+zinc groups (P <0.05, Fischer exact test).

TABLE 4

Birth weights per supplementation group.

	Supplementation groups			
	Control	β-Carotene +zinc	β-Carotene	Zinc
Normal, term delivery (n)[1]	38	42	42	42
Sex ratio (M:F)	26:12	27:15	22:20	23:19
Birth weight (kg)	3.2 (± 0.4)	3.3 (± 0.5)	3.1 (± 0.4)	3.1 (± 0.5)
Birth weight boys (kg)	3.2 (± 0.4)	3.4 (± 0.3)[2]	3.1 (± 0.4)	3.0 (± 0.6)
Birth weight girls (kg)	3.1 (± 0.5)	3.0 (± 0.6)	3.1 (± 0.5)	3.1 (± 0.4)
boys ≤3.0 kg	10	4[3]	13	12
boys ≤2.5 kg	2	0	3	4
girls ≤3.0 kg	6	9	11	9
girls ≤2.5 kg	2	3	2	3

[1] Excluding twins, preterm, stillborn and fatal congenital abnormalities.

[2] Different from the β-carotene and the zinc group, P< 0.05 (ANOVA)

[3] Different from all other groups, P< 0.01 (chi-square)

Prior to comparing birth weight among the groups, all twins, preterm infants, stillbirth and fatal congenital abnormalities were excluded from these analysis as these conditions can affect birth weight. Although there was no significant difference in mean birth weights among the groups, the birth weights of the boy infants was significantly different among the groups (P <0.05, ANOVA), with the β-carotene+zinc group being significantly higher (P <0.05) than the zinc group and almost significantly higher (P= 0.054) than the β-carotene group (**Table 4**). This is also apparent in the frequency distributions of the birth weights of the boy infants (**Figure 2**). Furthermore, there were significantly less boys with a birth weight of 3.0 kg or less in the β-carotene+zinc group as compared to all other groups (P <0.01, chi-square). Birth weight of girl infants was lower, but not significantly so, than that of boys, and did not differ among the groups (**Figure 3**).

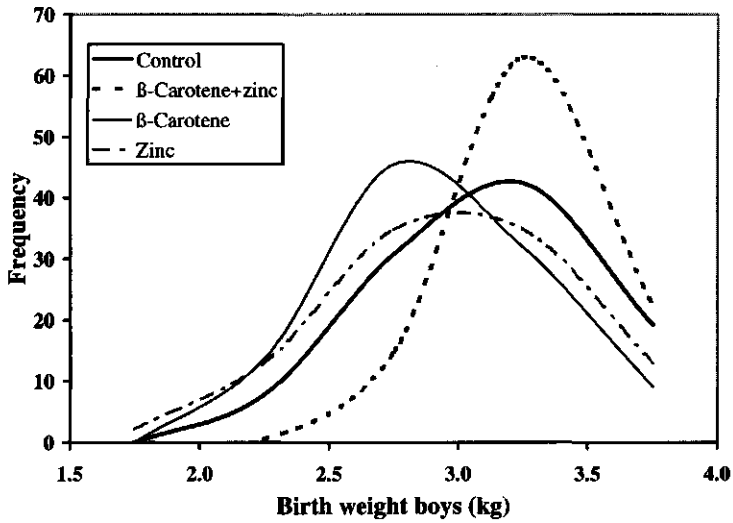

Figure 2. Birth weight frequency distribution curves (smoothed) of boy infants born of mothers supplemented during pregnancy with different micronutrients added to iron and folic acid supplementation. Control group received only iron and folic acid

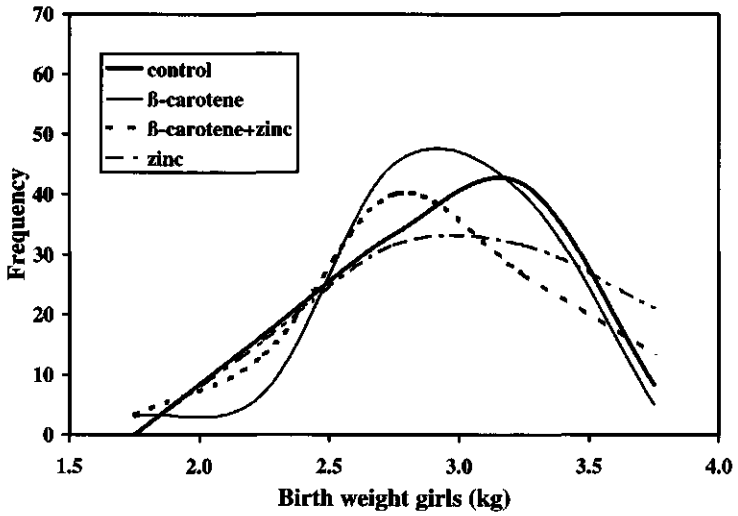

Figure 3. Birth weight frequency distribution curves (smoothed) of girl infants born of mothers supplemented during pregnancy with different micronutrients added to iron and folic acid supplementation. Control group received only iron and folic acid.

DISCUSSION

In this study, supplementation of pregnant women with not only iron and folic acid, but also β-carotene and zinc, increased birth weight, but only of boys. However, supplementation of pregnant women with the addition of only zinc to iron and folic acid increased the incidence of complications during delivery. There was no clear difference in micronutrient status indicators at 8 mo of pregnancy among the different supplementation groups although the decrease in plasma ferritin concentrations was lowest in the women receiving β-carotene and zinc combined, and β-carotene supplementation increased plasma concentrations of β-carotene concentrations but not of retinol. The progressive hemodilution during pregnancy complicates the interpretation of plasma concentrations of micronutrient status indicators however, and may have obscured effects of supplementation on biochemical indicators.

Micronutrient deficiencies are likely to occur concurrently, as dietary risk factors for deficiency are the same for many micronutrients. Earlier, we have shown that iron, vitamin A and zinc deficiency are prevalent in lactating mothers and infants in this area, and that vitamin A deficient mothers and infants have an increased risk of also being deficient for iron and/or zinc (13). In this perspective, the addition of only zinc or only β-carotene to the standard iron and folic acid supplementation might not address all the needs of pregnant women. This is supported by the finding in the

present study that only the combination of β-carotene and zinc but not of the single addition of either zinc or β-carotene had beneficial effects on birth weight.

Why this effect is only apparent in boys is not clear. However other studies have also reported that zinc supplementation improves growth more in boys than in girls (15, 16), and some animal studies have shown a greater sensitivity of males to zinc deficiency than females (17). These findings may be related to the higher growth rate of males, or to differences in body composition between males and females.

Besides direct effects of the supplemented micronutrients, interactions between micronutrients may also play an important role. Alleviation of one micronutrient deficiency might concomitantly increase the requirements for other micronutrients. In the present study, women receiving only zinc had the largest decrease during pregnancy in plasma β-carotene concentrations, pointing perhaps to an active role of zinc in vitamin A metabolism, and a greater utilisation of β-carotene with better zinc status (8, 18). The decrease in plasma ferritin concentrations was the least in the women receiving β-carotene and zinc, possibly reflecting the interactions between vitamin A, iron and zinc metabolism (19). Although the exact mechanisms are not known yet, the findings of the present study clearly indicate that multimicronutrient supplementation not only has additive but also synergistic effects.

The higher delivery complication rate, and the possibly increased incidence of neonatal complications in the zinc group, may be due to inadvertent interactions between micronutrients. Supplementation with zinc can impair iron absorption, and may compromise iron status (7). In this respect, it is important to note that hemoglobin and plasma ferritin concentrations at 8 mo of pregancy were lowest in the zinc group, and plasma ferritin concentrations during pregnancy decreased most in this group, although the differences failed to reach statistical significance. Although the exact mechanisms underlying the higher complication rates in the women who were supplemented with zinc cannot be elucidated here, and other factors may also play a role, the findings of this study certainly warrant concern.

The nutritional status of pregnant women is a major factor determining pregnancy outcome, including birth weight, pregnancy complications, and health and nutritional status of the newborn. Supplementation of iron and folic acid during pregnancy is standard practice in many countries. However, in developing countries deficiencies of other micronutrients are also prevalent. Supplementation of a wider range of micronutrients than only iron and folic acid during pregnancy not only ensures an adequate supply of lacking or scarce micronutrients, but also facilitates optimal utilization of supplemented micronutrients. By supplementing pregnant women with a balanced and complete array of micronutrients, effectiveness of supplementation can be enhanced by synergistic interactions, whereas possible antagonistic or inhibitory interactions can be compensated for. This study shows that the addition of β-carotene and zinc combined can augment the standard iron and folic acid supplementation of pregnant women, and improve pregnancy outcome.

ACKNOWLEDGEMENTS

We would like to thank all the mothers and the infants, the health volunteers and midwifes who participated in this study, and we are grateful for the enthusiastic support we received from Dr.Hendra, Dr. Anni and their staff. Furthermore we would like to thank the field team of Puslitbang Gizi, and ibu Asih and ibu Ndari from the laboratory of SEAMEO, Jakarta for their untiring efforts. Financial support for this study was received from the Netherlands Foundation for the Advancement of Tropical Research (WOTRO), and Ter Meulen Fund (Royal Netherlands Academy of Arts and Sciences).

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Zinc in Plants.

Metals can have profound effects on plants. Lack of zinc in the soil for example, will greatly reduce plant growth. Zinc deficiency is the most widely occurring micronutrient disorder affecting crops. Moreover, high level of phosphate fertilisers used with modern high yielding cereal varieties can exacerbate or induce zinc deficiency in crops. High levels of soil zinc also inhibit plant growth however, although certain plants grow only on soils very rich in certain metals. Indeed, in early times, the aspect of vegetation was an important tool in the search for metal ore. In 1809, Lejeune proposed that a subtype of pansy found only on zinc-rich meadows be named Viola zinci. In 1911, Heimans observed that this pansy occurred in The Netherlands only near the river Geul, and only where silt is deposited during winter. He also noted that this was probably due to the high concentration of zinc in the silt originating from the zinc mines upstream in Belgium. ("Het heet, dat de Geul van boven uit Belgie inderdaad zink in zijn water meevoert; dat blijft bij overstromingen op de oevers achter. Ge vindt in Nederland geen enkel geel zink-viooltje, dat niet aan de Geul staat"). Plants that grow on soils rich in heavy metals have been named "heavy-metal tolerating plants". As these plants also grow on normal soil but significantly less well than non-tolerant plants, it is likely that the greater competition on normal soils inhibits their wider distribution.

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CHAPTER 9

β-Carotene Supplementation Only Improves Vitamin A Status when Given in Combination with Zinc.

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Submitted for publication.

ABSTRACT

Background. Deficiencies of vitamin A, iron and zinc are prevalent in pregnant and lactating women and their infants in developing countries. Combined supplementation of several micronutrients during pregnancy could benefit both mother and infant. The effectiveness of adding β-carotene and zinc to the standard iron and folic acid supplementation during pregnancy in improving vitamin A status of mothers and infants 6 months post-partum was investigated.

Methods. After supplementation during pregnancy with β-carotene and zinc in a randomised, double-blind controlled trial, 170 mothers with their newborn infants were enrolled for follow-up 6 months post-partum.

Findings. Supplementation during pregnancy with β-carotene and zinc increased plasma retinol concentrations of both mothers and infants 6 months post-partum, and reduced the prevalence of vitamin A deficiency of infants at 6 months of age by more than 30%. Breast milk retinol concentrations were significantly higher in the β-carotene+zinc group at 6 months post-partum. β-Carotene supplementation during pregnancy without zinc did not improve vitamin A status in mothers or infants.

Interpretation. β-Carotene improved vitamin A status of mother and infant only when given in combination with zinc. This points to a specific role of zinc in the conversion of β-carotene to retinol. Impaired bioconversion of provitamin A carotenoids in zinc deficiency could explain in part the high prevalence of vitamin A deficiency in the presence of abundant dietary carotenoids as encountered in many developing countries. The addition of β-carotene and zinc to standard iron supplements could be an effective way to improve health of mothers and infants.

INTRODUCTION

Micronutrient deficiency during infancy can lead not only to poor growth performance, but also to increased risk of morbidity and mortality from infectious diseases, and delayed psychomotor development (1-4). Maternal nutritional status is the one of the most important factors determining the nutritional status of infants (5). The nutritional status of women during pregnancy determines to a large extent the nutritional stores with which infants are born, and the nutrition status of women post-partum also determines breast milk micronutrient content. In many developing countries, deficiencies of various micronutrients are prevalent in pregnant and lactating women, and also in their infants. Micronutrient supplementation during pregnancy has the advantage of addressing the needs of both mothers and infants simultaneously.

Vitamin A deficiency is still a major health problem worldwide. Vitamin A supplementation has been shown to reduce mortality in children under the age of 5 years by an estimated 23-34% (6). Several approaches to reduce vitamin A deficiency have been implemented, including intermittent high dose vitamin A supplementation of infants and children, and single high dose vitamin A supplementation of the mother directly post-partum. However, because of the teratogenicity of vitamin A, only small doses (<10,000 IU/day or <25,000 IU/week) can be given safely during pregnancy. In addition, only small doses of vitamin A can be safely given to young infants (7). β-Carotene is a precursor of vitamin A, and is not teratogenic. Therefore it is considered a useful alternative to retinol for supplementation during pregnancy. β-Carotene is also the main dietary source of vitamin A in developing countries, but both the uptake and the conversion of dietary β-carotene to retinol are less efficient than previously thought (8).

Iron deficiency is the most prevalent micronutrient deficiency, and over 50% of the pregnant women in developing countries are estimated to be iron deficient (9). Supplementation of iron, in combination with folic acid, during pregnancy has been implemented as a standard program in many countries. The same dietary factors leading to iron deficiency also lead to zinc deficiency, suggesting that zinc deficiency is very prevalent also.

If more than one micronutrient is deficient, supplementation of only one micronutrient will not adequately address all needs. Moreover, utilisation of the supplemented micronutrient can be compromised by the presence of deficiency of other micronutrients, reducing the effectiveness of supplementation. For example, the prevalence of anaemia can be reduced even more by supplementation with vitamin A in addition to iron (10).

This study investigates whether supplementing women during pregnancy with β-carotene and zinc, in addition to iron and folic acid, can improve vitamin A status of mothers and newborns 6 months post-partum.

SUBJECTS AND METHODS

Study design and location.

The study was designed as a randomised, double-blind, controlled supplementation trial with pregnant women. Pregnant women (229) with a gestational age <20 weeks had been recruited from 13 adjacent villages in a rural area in Bogor District, West Java, Indonesia. All women were supplemented with iron and folic acid (30 mg of iron as ferrous fumarate and 0.4 mg of folic acid per day). In addition, one group of women received β-carotene (4.5 mg per day), one group zinc (30 mg of zinc as sulphate per day), and one group β-carotene plus zinc (4.5 mg and 30 mg per day, respectively). Supplements were prepared by the pharmacy of the Gelderse Vallei Hospital (Bennekom, The Netherlands), given as capsules, and taken daily until delivery. The effects of supplementation on pregnancy outcome will be reported elsewhere.

Subjects and procedures.

All women supplemented during pregnancy were eligible after delivery for follow-up of infant and mother until 6 months post-partum. The women had been informed at the beginning of supplementation and written informed consent was given. Exclusion criteria at enrolment were twin pregnancy because of a different growth pattern in twin infants, and congenital abnormalities interfering with growth, development or metabolism. Mothers and infants were assessed anthropometrically each month. Two breast milk samples were collected from each mother; one in the first and one in the sixth month post-partum. Furthermore, a blood sample (5 mL) was collected at the end of the study of both the mother and the infant for assessment of micronutrient status.

Methods.

Anthropometry included measurement of weight and length by trained anthropometrists using standard methods. Non-fasting, morning blood samples were collected using a closed-tube vacuum system to avoid zinc contamination (Becton and Dickinson. Leiden, The Netherlands). Blood samples were immediately stored at 4°C to prevent microhaemolysis and separated within 5 h. Plasma and serum samples were aliquoted and stored at -30°C until analysis.

Haemoglobin concentrations were measured by a standard cyanoblue method (Humalyzer, Germany). Serum zinc concentrations were analysed with flame atomic absorption spectrophotometry (Varian, Clayton South, Vic, Australia) using trace-element free procedures, as described in an earlier paper (5). The CV (10% duplicate analysis and pooled control samples) for zinc analyses was <5%. Plasma retinol and β-carotene concentrations were measured by HPLC, with two separate extractions and separations as described by Ehrhardt et al. (11). The CV for retinol and carotenoid analyses (10% duplicate analysis and pooled control samples) was <5%. Breast milk retinol and carotenoid concentrations were measured as described by Jackson et al. (12). In short, 200 L of breast milk was mixed with KOH-ethanol (12.5% w/v, 250 L), and internal standard (9 mol/L echinenone in ethyl alcohol, 100µL) was added. The mixture was saponificated at 45°C for 2 h. Retinol and carotenoids were doubly extracted with hexane, dried under nitrogen and reconstituted in solvent for HPLC

analysis. Creamatocrit was measured to estimate breast milk fat content using a method analogous to haematocrit measurement, and breast milk fat content was calculated according to Lucas et al. (13). The CV for the measurement of breast milk fat content and concentrations of retinol and β-carotene was <10%. Ferritin was measured using a commercial ELISA kit (IBL-Hamburg, Germany) according to the guidelines of the manufacturer. α1-Acid glycoprotein (AGP) was measured using immunoturbidimetric techniques at the Northern Ireland Centre for Diet and Health, University of Ulster, Northern Ireland (Cobas Fara analyzer, Roche Products, Welwyn, UK). The CV for the ferritin and AGP assays was <10%.

Ethical approval.

The protocol was approved by the Ethical Committees of the National Health Research and Development Institute of Indonesia and of the Royal Netherlands Academy of Arts and Sciences.

Statistical analysis.

Data was checked for normal distribution using the Kolmogorov-Smirnov test of normality. Plasma ferritin concentrations and breast milk concentrations of retinol, β -carotene and zinc were transformed to logarithms before statistical analyses. Differences in prevalence were tested with Pearson's chi-square test. Differences among groups were tested with analysis of variance (ANOVA), with sex as between-subject factor when necessary, or Kruskal Wallis for non-parametric data. Differences in biochemical indicators among supplementation groups were analysed using ANOVA or analysis of covariance (ANCOVA), with AGP as covariate. Correlations between micronutrient status of mothers and infants were investigated with multiple linear regression, controlling for age, sex and AGP when necessary, or Spearman's rank test for non-parametric data. Decreases in breast milk concentrations between 1 months and 6 months of lactation were tested with paired t test and repeated measurements multivariate ANOVA. When the overall F test was significant, differences among the groups were further investigated with post-hoc multiple comparisons for ANOVA, and in a general linear model for ANCOVA. Frequency distribution curves were smoothed using a moving average, taking the two previous and two following values into account. Anthropometrical Z scores were calculated with EPI-Info, Version 6.04b (CDC, Atlanta GA, USA), and statistical analysis was carried out with the SPSS 7.5.2 (SPSS Inc, Chicago, IL, USA) software package.

RESULTS

Of the 179 women who were supplemented during pregnancy, 170 were enrolled after delivery with their newborn infants for follow-up for 6 months. The infants of nine mothers were not enrolled for follow-up because of twin pregnancy (3x), still birth (1x) or neonatal death (5x). The mothers had been supplemented during pregnancy with iron and folic acid (control group), iron and folic acid combined with β -carotene (β-carotene group), iron and folic acid combined with zinc (zinc group), or iron and folic acid combined with both β-carotene and zinc (β-carotene+zinc group). Infants were born between November 1998 and July 1999. During follow-up, 34 mother-infant pairs dropped out for various reasons (**Figure 1**): 2 moved house, 29 withdrew from the study, 1 infant died, 1 mother was too ill with tuberculosis to attend

follow-up, and 1 infant developed protein-energy malnutrition and was withdrawn from the study for treatment.

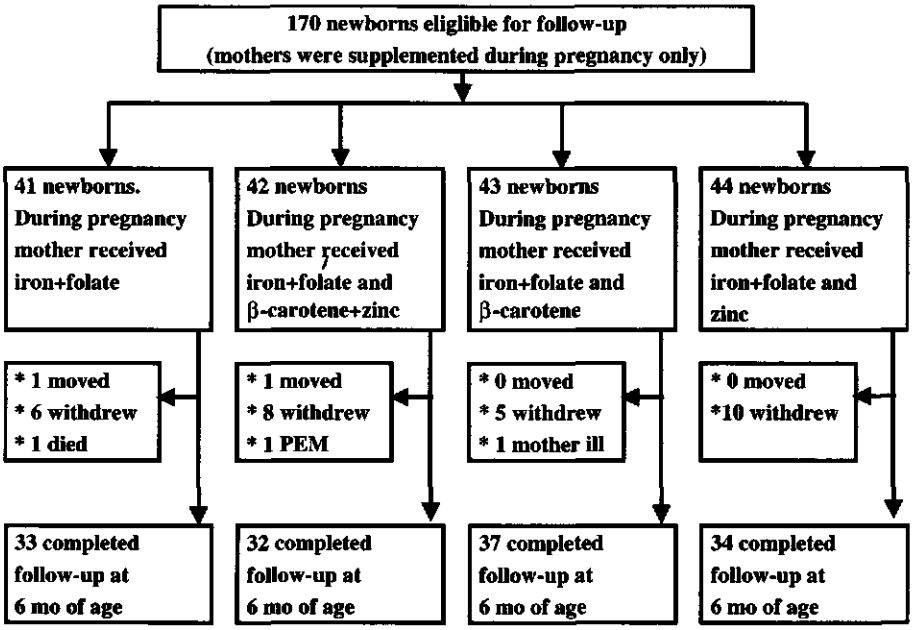

Figure 1. Trial Profile

Newborn infants were evenly born into the 4 groups (**Table 1**). There were 101 boys and only 69 girls, but both sexes were equally distributed over the groups. Seven infants were born preterm (**Table 1**). There was no significant difference in birth weight among the groups when taking both sexes together, but the birth weight of boys in the β-carotene+zinc group was significantly higher than in the β-carotene group and the zinc group ($p<0.05$, ANOVA).

At the age of 6 months, there were no significant differences in anthropometric parameters (expressed as Z scores) among the groups (**Table 2**). There was however a strong positive correlation between birth weight and Z scores at 6 months of age for both weight for age ($R=0.28$, $P< 0.01$, multiple linear regression controlling for sex) and height for age ($R=0.28$, $P<0.001$, multiple linear regression controlling for sex), but not for weight for height.

	Supplementation groups[1]			
	Control	**β-Carotene +zinc**	**β-Carotene**	**Zinc**
n	41	42	43	44
Sex ratio (M:F)	27:14	27:15	23:20	24:20
Preterm	3	1	1	2
Birth weight (kg)[2]	3.1 (± 0.5)	3.2 (± 0.5)	3.0 (± 0.4)	3.0 (± 0.5)

[1] No differences between the groups for any of the baseline characteristics, except for the birth weight of boys in the β-carotene + zinc group which was significantly higher than the β-carotene group and the zinc group ($P<0.05$, ANOVA)

[2] Mean (± SD)

Table 1. General characteristics of the newborn infants at birth.

	Supplementation groups			
	Control	**β-Carotene +zinc**	**β-Carotene**	**Zinc**
n	33	32	37	34
Sex ratio (M:F)[1]	21:12	22:10	20:17	19:15
Age (mo)[2]	6.0 (± 0.6)	6.1 (± 0.8)	6.0 (± 0.6)	6.3 (± 0.7)
Anthropometry (Z-scores)[2]				
Weight for age	-0.06 (± 0.76)	-0.06 (± 0.74)	-0.28 (± 0.92)	-0.28 (± 0.88)
Height for age	-0.81 (± 0.73)	-0.80 (± 0.71)	-0.78 (± 0.64)	-0.70 (± 0.76)
Weight for height	0.68 (± 0.73)	0.66 (± 0.83)	0.36 (± 0.64)	0.28 (± 0.95)

No significant differences between the groups [1](chi-square) [2] (ANOVA).

Table 2. Anthropometry of the infants at 6 months of age.

In the women supplemented during pregnancy with β-carotene and zinc, plasma retinol concentrations were significantly higher than in all other groups ($P<0.05$, ANCOVA controlling for AGP, **Table 3**). Also, the infants of these mothers

had plasma retinol concentrations significantly higher than the infants from the other groups (P<0.01, ANCOVA controlling for AGP). Plasma β-carotene concentrations were also significantly higher in the women who were supplemented with β-carotene and zinc during pregnancy as compared to the other groups (P<0.01, Kruskal Wallis). At 6 months post-partum, plasma concentrations of retinol and β-carotene of the mothers were significantly correlated with those of their infants (P<0.01, R=0.23 multiple linear regression controlling for AGP concentrations of mothers and infants, and P<0.01, ρ=0.28, Spearman's rank respectively, **Table 3**). The haemoglobin concentrations and the plasma ferritin and zinc concentrations did not differ among the groups.

	Supplementation groups			
	Control	**β-Carotene +zinc**	**β-Carotene**	**Zinc**
n	33	32	37	34
Concentrations[1]				
Haemoglobin (g/L)				
Mothers	122 (± 11)	123 (± 11)	120 (± 13)	120 (± 9)
Infants	109 (± 15)	111 (± 10)	108 (± 10)	111 (± 10)
Plasma ferritin (μg/L)				
Mothers	21.6 (6.7-39.1)	19.0 (9.7-38.4)	16.8 (8.7-26.5)	20.7 (10.2-29.7)
Infants	23.1 (11.4-41.6)	38.2 (11.7-61.3)	24.7 (8.2-48.0)	24.1 (9.4-61.8)
Plasma retinol (μmol/L)				
Mothers[2]	0.90 (± 0.31)	1.12 (± 0.34)[4]	0.89 (± 0.27)	0.93 (± 0.39)
Infants[2]	0.62 (± 0.18)	0.77 (± 0.18)[5]	0.60 (± 0.16)	0.62 (± 0.16)
Plasma β-carotene (μmol/L)				
Mothers[3]	0.14 (0.11-0.19)	0.26 (0.18-0.36)[6]	0.15 (0.10-0.29)	0.17 (0.07-0.27)
Infants[3]	0.03 (0.00-0.07)	0.04 (0.00-0.08)	0.03 (0.00-0.08)	0.03 (0.00-0.05)
Plasma zinc (μmol/L)				
Mothers	11.7 (± 1.3)	11.5 (± 1.8)	11.4 (± 1.4)	11.4 (± 1.4)
Infants	12.4 (± 2.1)	12.9 (± 2.0)	12.3 (± 2.1)	12.4 (± 2.5)

[1] Mean (± SD) or median (IQR); Ferritin concentrations were transformed to logarithm before statistical analy
Correlation between mother and infant: [2] p<0.01, R=0.23 (multiple linear regression controlling AGP);
[3] p<0.01, ρ=0.48 (Spearman's rank test).
Significantly different from other groups: [4] p<0.05 and [5] p<0.01 (ANCOVA controlling for AGP); [6] p<0.01 (Kruskal Wallis).

Table 3. **Indicators of micronutrient status of mothers and their infants, 6 months post-partum.**

The effect of supplementation during pregnancy on plasma retinol concentrations of mothers and infants 6 months post-partum is clearly illustrated in the frequency distributions (**Figure 2 and 3**). The distribution curve of plasma retinol concentrations 6 months post-partum of the mothers in the β-carotene+zinc group is shifted to higher concentrations, as compared to all other groups (**Figure 2**). The distribution curve of women who received β-carotene is also shifted to the right, but to a much lesser extent than the β-carotene+zinc group. The distribution curve of plasma retinol concentrations of the infants born from mothers receiving β-carotene+zinc during pregnancy is also clearly shifted towards higher retinol concentrations, whereas the distribution curve of the infants in the β-carotene group is in between the control and β-carotene+zinc groups (**Figure 3**).

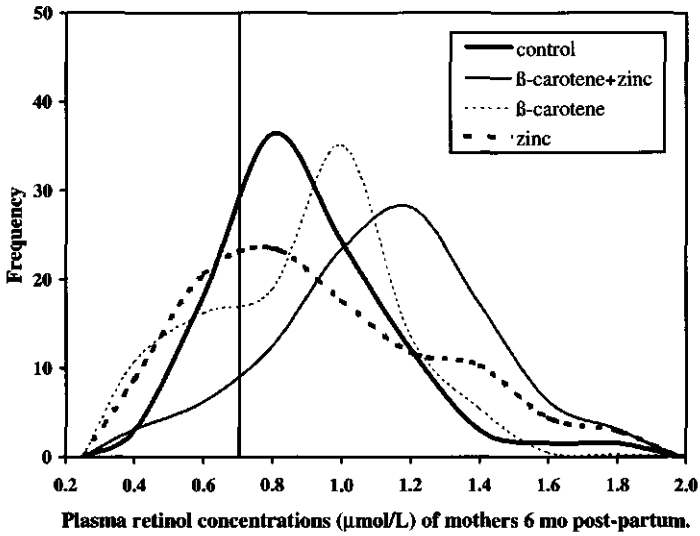

Figure 2. Frequency distribution curve (smoothed) of plasma retinol concentrations 6 months post-partum in mothers, supplemented during pregnancy with β-carotene and zinc in addition to iron and folic acid. The control group received only iron and folic acid.

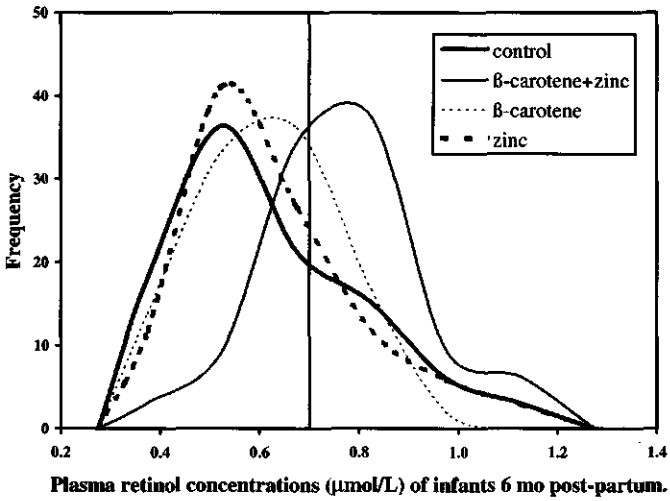

Figure 3. Frequency distribution curve (smoothed) of plasma retinol concentrations 6 months post-partum in infants, born of mothers supplemented during pregnancy with β-carotene and zinc in addition to iron and folic acid. The control group received only iron and folic acid during pregnancy.

Supplementation of pregnant women with β-carotene and zinc during pregnancy decreased the prevalence of vitamin A deficiency in the newborns 6 months post-partum by more than 30% (41% in the β-carotene+zinc group vs. 73-74% in the other groups, P<0.01, chi-square). In mothers, the prevalence of vitamin A deficiency was lower, hence the effect of supplementation was less pronounced, and the difference among the groups was not statistically significant. Still, in the mothers who received β-carotene and zinc, the prevalence of vitamin A deficiency 6 months post-partum was 9%, whereas in the other groups the prevalence of vitamin A deficiency ranged from 21% (control group) to 32% (zinc group).

In the first month of lactation, the micronutrient concentrations and fat content of breast milk were not different among the groups (**Table 4**). Breast milk concentrations of retinol and zinc decreased significantly between the first and sixth months of lactation in all groups (P<0.001, paired t test). At 6 months post-partum retinol concentrations in breast milk were significantly higher in the women supplemented during pregnancy with β-carotene and zinc than the women in the control group (P<0.05, ANOVA). Breast milk β-carotene concentrations 6 months post-partum were significantly higher in both the women who were supplemented during pregnancy with β-carotene and zinc as well as with β-carotene alone as compared to the control group (P<0.01, ANOVA).

	Supplementation groups			
	Control	β-Carotene +zinc	β-Carotene	Zinc
n	33	32	37	34
Breast milk concentrations				
Fat (g/L)				
1 mo	30.6 (± 12.1)	31.6 (± 9.2)	34.8 (± 12.0)	32.3 (± 15.3)
6 mo	33.8 (± 14.0)	28.8 (± 11.0)	31.8 (± 16.0)	34.2 (± 16.5)
Retinol (nmol/g fat)[2]				
1 mo	25.3 (21.2-44.3)	27.6 (15.8-53.6)	26.3 (17.8-36.5)	26.4 (21.9-41.7)
6 mo	13.9 (9.5-18.3)	19.7 (12.5-29.0)[3]	14.9 (9.7-23.2)	15.2 (9.9-24.0)
β-Carotene (nmol/g fat)				
1 mo	0.29 (0.16-0.58)	0.30 (0.20-0.69)	0.36 (0.20-0.57)	0.26 (0.15-0.67)
6 mo	0.28 (0.17-0.36)	0.41 (0.32-0.69)[4]	0.43 (0.26-0.67)[4]	0.37 (0.24-0.45)
Zinc (μmol/L)[2]				
1 mo	40.2 (29.0-49.8)	47.7 (37.0-64.1)	45.6 (34.8-59.8)	49.9 (31.5-63.9)
6 mo	17.0 (12.0-26.1)	18.4 (13.2-29.4)	18.4 (11.9-29.0)	18.5 (11.9-29.8)

[1] Means (± SD) or median (IQR). Concentrations of retinol, β-carotene and zinc were transformed to logarithms before statistical analysis. [2] Significant decrease from 1 mo to 6 mo ($P < 0.01$, paired t test). Significantly different from control group: [3] $p<0.05$ and [4] $p<0.01$ (ANOVA)

Table 4. Micronutrient concentrations in breast milk samples at 1 month and 6 months post-partum

DISCUSSION

This study shows that β-carotene supplementation is only effective in improving vitamin A status when given in combination with zinc. This effect of zinc on the conversion of β-carotene to retinol has not been reported previously. In the present study, supplementation of pregnant women during pregnancy with β-carotene and zinc increased plasma retinol concentrations of both mothers and infants at 6 months post-partum, and reduced the prevalence of vitamin A deficiency of the infants by more than 30%. Intriguingly, β-carotene supplementation alone, without zinc, did not significantly increase plasma retinol concentrations neither in the mothers nor in the infants. Analogous to plasma, retinol concentrations in breast milk at 6 months post-partum were significantly higher in the women supplemented during pregnancy with β-carotene and zinc, but not in the women who received only β-carotene.

The findings of this study point to a specific role of zinc in the conversion of β-carotene to retinol, distinct from the role of zinc in the mobilisation of vitamin A as reported by other studies, which have shown that zinc deficiency can reduce plasma retinol-binding-protein concentrations (14,15). All women who received β-carotene had higher breast milk concentrations of β-carotene 6 months post-partum than the

control group. The increased breast milk concentrations of β-carotene 6 months after β -carotene supplementation is most likely a direct result of higher concentrations of β-carotene in the fat tissue of the breast, as β-carotene is primarily stored in fat tissue. However, only the women who also received zinc in addition to β-carotene had increased concentrations of retinol in plasma and in breast milk. Thus, the β-carotene supplement would appear to be utilised for conversion to retinol only when zinc is also given. The conversion of β-carotene to retinol is mainly via central enzymatic cleavage by 15-15'-dioxygenase, with a likely role of metals in its activity (16). Main sites of cleavage are thought to be the gut and the liver, but the enzyme is expressed in most tissues (17). Interestingly, plasma concentrations of β-carotene 6 months post-partum were increased in the women who received zinc in addition to β-carotene, but not in the women who received only β-carotene. Apparently, zinc is also important for the mobilisation of β-carotene from tissue storage to plasma, but not to breast milk.

There are several possible explanations for the improved vitamin A status in those infants born from mothers who received zinc in addition to β-carotene during pregnancy. Increased neonatal vitamin A stores at birth could have made an important contribution. Foetal vitamin A stores are accumulated during the last trimester of pregnancy, but depend on maternal plasma retinol concentrations (18). Although colostrum and early milk always contain high concentrations of retinol, after a few weeks of lactation the concentrations fall, and the high requirements of rapidly growing infants may no longer be met. In the present study the breast milk of women supplemented during pregnancy with β-carotene and zinc contained significantly more retinol and β-carotene after 6 months of lactation. The contribution of the higher breast milk retinol concentrations towards maintaining vitamin A nutriture in these infants can be estimated. If we assume an average daily milk intake of 600 ml, and an average fat content of breast milk of 31 g/L, it then follows that the median daily retinol intake from breast milk of the infants in the β-carotene+zinc group was 105 IU [IQR 66-154], whereas the median daily intake of the infants in the control group was only 75 RE [IQR 50-97]. For comparison, the UK lower reference nutrient intake (LRNI) for retinol in this age group is 150 RE (19).

The contribution of breast milk β-carotene to the vitamin A status of the infants is probably small. Following the same calculation as for retinol, the median daily intake of β-carotene from breast milk of the infants in the β-carotene group was only 7 RE [IQR 4-11], assuming a conversion factor of 1:6. Furthermore, the infants in the β-carotene group do not have significantly increased plasma retinol concentrations, although breast milk β-carotene concentrations were also high. Thus it is not very likely that an increase in breast milk β-carotene concentrations alone can contribute substantially to improving vitamin A status in the infants.

In the body, β-carotene can be converted into retinol, and when provided in oil approximately 2.4 μg of β-carotene can yield 1 μg of retinol (20). However, the bioavailability of β-carotene from fruits and vegetables is much lower, and estimates of the extent of conversion of β-carotene to retinol have varied widely among studies (21,22). On the basis of the available data, the US Institute of Medicine recently revised the factor for calculating the amount of β-carotene in a mixed diet required to

supply 1 μg of retinol. This amount is now estimated to be 12 μg of β-carotene, whereas formerly it was only 6 μg (23). Differences in zinc status may in part explain the wide range of estimates of the extent of conversion. In developing countries, most vitamin A is derived from provitamin A carotenoids, especially β-carotene. Impaired bioconversion of pro-vitamin A carotenoids to retinol due to zinc deficiency could partly explain the high prevalence of vitamin A deficiency in the presence of apparently abundant dietary carotenoids. In Nepal, supplementation with either vitamin A or β-carotene during pregnancy was shown to increase plasma retinol concentrations in infants at the age of 3 months, but supplementation with β-carotene was much less effective than with vitamin A (24). Concurrent zinc deficiency could well have reduced the effectiveness of the β-carotene supplementation.

In Bangladesh, supplementation of women directly post-partum with a single high dose of vitamin A (200.000 IU) or daily supplementation with β-carotene during lactation did not increase serum retinol concentrations of the mothers nor the infants at 6 months post-partum (25). In the present study, retinol concentrations were increased in the plasma of both the mother and infants, as well as in breast milk at 6 months post-partum, after supplementation of β-carotene and zinc during pregnancy. Hence, supplementation during pregnancy appears to be more effective and has more benefits for both mother and infant than supplementation of the mother post-partum.

The findings of this study clearly show that β-carotene can only improve vitamin A status when zinc is also available. Supplementation during pregnancy with β -carotene and zinc, but not with β-carotene alone, is effective in improving the vitamin A status of both the mother and the infant at 6 months post-partum. Increased breast milk vitamin A content is an important contributor to this effect. In view of the high prevalence of vitamin A deficiency in infants and lactating mothers in developing countries, and the direct relationship between vitamin A deficiency and increased morbidity and mortality, supplementing pregnant women with β-carotene and zinc is clearly indicated. Moreover, as iron supplementation of pregnant women is widely implemented, the addition of β-carotene and zinc to these supplements could be a very efficient and cost-effective measure to improve health of both infants and lactating mothers.

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Publication Nightmares.

Even though today the writing of a scientific publication is still a major task, this bears no comparison to the amount of work and frustration that earlier scientists had to face. Georg Rumphius for instance was a German medical doctor and botanist who worked on Ambon, from 1653 until his death in 1702. He went to Indonesia in the service of the VOC, but was eventually relieved of his work as a merchant because of the scientific importance of his voluntary botanical work. He became known as 'Plinius Indicus' as he systematically studied and described the unique flora and fauna of the Maluccu Islands. His work is still of relevance today. He became blind during the writing of his six-volume work on the botany of the Maluccu Islands, "Amboinsch Kruid-boek" or "Herbarium Amboinense". He continued working on the manuscript however with the aid of hired artists. When almost finished, at least half the illustrations were destroyed in a fire. When the manuscript was completed at last, it was sent to The Netherlands for publishing, but the manuscript was lost in a battle at sea, when the ship was attacked and sunk by the French in 1692. However, Rumphius succeeded in submitting a second version, 5 years later, based on copies. For unknown reasons, his work was only published in 1750, 48 years after his death. Another great biologist working in Indonesia, whose work is also still of relevance today, was Pieter Bleeker, a Dutch army doctor interested in fish. He arrived in Batavia in 1842, and in the following 30 years wrote the nine volumes of the "Atlas Ichthyologique". It took 18 years before the first volume was published. During his life he sent more than 12,000 fish specimen from Indonesia to The Netherlands. Due to his death in 1878, volume 9 remains unfinished.

Rumphius GE. Het Amboinsch Kruid-boek, and Amboinsche Rariteiten-kamer. Meinarduytwerf, Amsterdam 1750. Bleeker P. Atlas Ichthyologique des Indes Orientales Neerlandaises. Muller, Amsterdam 1862-1878.

CHAPTER 10

General Discussion.

INTRODUCTION

The main objective of the research described in this thesis was to investigate the effects of supplementation with β-carotene, iron and zinc on micronutrient status, growth and immune function. First the prevalence of vitamin A, iron and zinc deficiency was assessed, as well as the interrelationship of these deficiencies with each other and with growth impairment and immune function. Special attention was given to the relationship between the nutritional status of the mother and her infant (Chapters 2 and 3). Subsequently, the effects of supplementation of infants with β-carotene, iron and zinc on micronutrient status and deficiency were investigated. Furthermore, the effects of supplementation on growth and immune function were studied (Chapters 4-6). In addition, the extent of changes in plasma concentrations of commonly used indicators of micronutrient status in the presence of inflammation, and implications for the assessment of micronutrient status were explored (Chapter 7). In a separate study, effects of supplementation of pregnant women with β-carotene and zinc in addition to iron and folic acid on pregnancy outcome were investigated. Mothers and newborns were followed for 6 months post-partum to study the effects of supplementation during pregnancy on micronutrient status of mothers and newborns post-partum, and growth of the new born during the first 6 months (Chapters 8 and 9).

METHODOLOGY AND DESIGN OF THE STUDIES

The first study was a cross-sectional survey in infants and their lactating mothers. This design is well suited to assess the prevalence of deficiencies of various micronutrients. Relationships, eg. between the micronutrient status of mothers and infants, can be established but cause and effect cannot, as only one point in time is studied, and there is no intervention. The same limitations apply to anthropometry, as growth is a continuos process, and a single anthropometrical assessment measures only the end-point of the preceding process of growth. The cross-sectional survey was carried out in the same area and the same age group as the two supplementation studies, so that baseline information on micronutrient status and growth was available for the two intervention studies.

The supplementation trial in infants was designed as a randomised, double-blind, placebo-controlled intervention study. It was initially designed as a 2x2 trial with β-carotene and zinc supplementation. However, at the request of UNICEF-Jakarta two groups were added to investigate the effects of iron and zinc supplementation also. The final design was a 6 cell trial centering upon the zinc and placebo groups; with one arm being a 2x2 trial with iron and zinc supplementation, and one arm being a 2x2 trial with β-carotene and zinc supplementation. Theoretically a 2x2x2 trial would have been preferable, but resources did not allow such an option. Growth and morbidity were monitored monthly. Blood samples however were only taken at the end of the supplementation study for three reasons. The first consideration is ethical; when a baseline blood sample is taken, there is an ethical obligation to treat anaemia, iron deficiency and/or vitamin A deficiency immediately, given the established and considerable health risks of these deficiencies. Infants with a known deficiency cannot be allocated at random to supplementation of possibly placebo. For zinc deficiency, this is at the moment less clear. However, treating deficiencies found at baseline would

mean exclusion of these infants from the study, and lead to not only a considerable drop out at recruitment, but also create a bias in the recruited infants, as only infants with no prior deficiency would be selected. Secondly, there was concern that taking a baseline blood sample would lead to considerable drop out prior to the second blood sample. Finally, during infancy biochemical indicators of micronutrient status and immune function change considerably. Hence, the informative value of a blood sample taken at 4 months of age is limited, and not strictly comparable with the end point blood sample taken at 10 months of age. However, with only an end point blood sample, there was some restriction in the possibilities of statistical analyses, e.g. subgroups and stratification, and in the interpretation of causality of some relationships. Morbidity has not yet been thoroughly addressed due to the difficulties of deriving quantitative parameters from the available data. However, these analyses will be carried out at a later stage.

The supplementation trial with pregnant women was designed as a randomised, double-blind intervention trial. Because the benefits of iron and folic acid supplementation during pregnancy have been well established, and iron and folic acid supplementation during pregnancy is national policy in Indonesia, a placebo group was considered unethical. Therefore, all women, including the control group, received the standard iron and folic acid supplementation. The design was a 2x2 trial in which β-carotene and/or zinc were supplemented in addition to iron and folic acid. Women were recruited at a gestational age of between 10 and 20 weeks, because a gestational age of less than 10 weeks was considered too early to be able to diagnose pregnancy with enough certainty, and later than 20 weeks of gestational age would diminish the duration of supplementation. Because effects of prenatal supplementation were studied, supplementation was until delivery only. The women were seen monthly for anthropometry and health assessment. Blood samples were taken from the women at recruitment and at 8 months of pregnancy. Delivery complications of the mother and the neonate and birth weight were recorded within 3 days of birth, using a card with pictograms, to be filled in and signed by the birth attendant or health volunteer. Newborns and their mothers were seen monthly, until 6 months post-partum. Growth and morbidity of the newborn was assessed monthly, and a blood sample was taken from the newborn and the mother 6 months post-partum. Furthermore, two breast milk samples were collected, 1 and 6 months post-partum respectively. Although 3 blood samples were taken from the women, the comparison of biochemical indicators over these three time points was hampered by the substantial physiological changes that occur during pregnancy. A major drawback of recruiting women when already pregnant is that not only the baseline biochemical, but also the anthropometrical measurements are already affected by the state of pregnancy. However, a study design covering all women in the fertile age in order to start recruitment and supplementation before conception, although much preferable, would require a huge number of subjects, not compatible with the available resources. From the newborns, only an endpoint blood sample was taken, for the same reasons and with the same limitations as for the supplementation trial in infants.

MAIN FINDINGS AND INTERPRETATION

Micronutrient status and deficiency.

The studies described in this thesis show that anaemia, iron deficiency, vitamin A deficiency and zinc deficiency are all prevalent in both pregnant and lactating women as well as in infants in Bogor district (Chapters 2, 4, 5, 8 and 9). Earlier studies in this area showed that anaemia, iron deficiency and vitamin A deficiency are prevalent in pregnant women and in children (1-3). In the present studies, we show that zinc deficiency is prevalent also. Furthermore, the studies described in this thesis show that vitamin A, anaemia, iron deficiency and zinc deficiency occur concomitantly, are correlated with each other, and that there is a significantly increased risk in vitamin A deficient subjects of being also deficient for iron and/or zinc. Deficiencies of various micronutrients during infancy and childhood increase the risk of morbidity and mortality of infectious diseases, can impair growth, and may result in delayed psychomotor development and impaired learning (4-8). In this context, the high prevalence of deficiencies of various micronutrients as found in the present studies in a population that is not extremely poor or disadvantaged, has access to primary health care facilities, and is apparently healthy, certainly warrants concern. Micronutrient deficiency may be a major factor underlying the prevailing high infant mortality (60 per 1000 infants) in Indonesia (9).

The high prevalence of deficiencies of various micronutrients in pregnant and lactating women is an important finding. Many studies have shown adverse consequences of micronutrient deficiency during pregnancy on pregnancy outcome including maternal and neonatal mortality, premature birth, and birth weight (10-13). Maternal mortality is still high in Indonesia (65 per 10,000 pregnancies), and could be associated with the high prevalence of deficiency of various micronutrients in pregnant women (9). However, the present studies were not designed to measure effects on mortality, either maternal or infant, as much larger numbers of subjects are needed for this.

Effects of supplementation on micronutrient status and deficiency.

In the studies described in this thesis, supplementation with iron was effective in reducing anaemia and iron deficiency in infants (Chapter 4). Supplementation with iron combined with zinc reduced the efficacy of the iron supplementation. However, the combined supplementation of iron and zinc was still effective in reducing the prevalence of anaemia and iron deficiency, whilst also reducing the prevalence of zinc deficiency. Supplementation with iron alone does not impair zinc status, but supplementation with zinc alone appears to have a slight negative effect on iron status. There has been major concern that supplementation with either iron or zinc alone could inhibit the uptake of the other micronutrient (14). However, it seems that the negative effects of supplementation with the single micronutrients are small, and that substantial inhibition is only seen when iron and zinc are given together in relatively high concentrations such as in a combined supplement. In pregnant women, supplementation with micronutrients did not alter the plasma concentrations of any of the micronutrient status indicators during pregnancy, probably because changes were obscured by the much larger effects of haemodilution (Chapter 8).

Zinc is thought to play a role in vitamin A metabolism (15,16). Supplementation of infants with β-carotene, with or without zinc, did not significantly improve plasma retinol concentrations, although the prevalence of vitamin A deficiency was somewhat reduced (Chapter 5). However, plasma β-carotene concentrations in infants increased substantially after supplementation with β-carotene. In pregnant women, supplementation with β-carotene combined with zinc during pregnancy improved vitamin A status of both mother and newborn 6 months post-partum, whereas supplementation with β-carotene alone had no effect (Chapter 9). This indicates that zinc plays an important role in the conversion of β-carotene to retinol. This is also illustrated by the finding that women who received only β-carotene supplementation, had increased breast milk concentrations of β-carotene but not of retinol. Also, these women had neither increased plasma retinol nor β-carotene concentrations, pointing to an impaired utilisation of the supplemented β-carotene in the absence of zinc. In this context, it is important to note the high prevalence of low plasma zinc concentrations in the lactating women (25% or more < 10.7 mol/L). The fact that the synergistic effect of zinc on β-carotene supplementation is not apparent in the infants can have several reasons. First of all, metabolic functions are often not yet fully developed in infants, so that the capacity of many enzymatic processes is less. This might reduce the potential contribution of β-carotene to vitamin A status. Also, infants have very little adipose tissue, hence infants have less capacity to store the supplemented β-carotene.

Supplementation with vitamin A and iron has been shown to be more effective in reducing the prevalence of anaemia than supplementation with iron alone (17). Although exact mechanism are still unknown, it is thought that vitamin A improves the utilisation of iron (18). In the present studies, supplementation of infants with β-carotene reduced the prevalence of anaemia (Chapter 5). However the effects were not very large, perhaps because the infants were supplemented with β-carotene and not with retinol. In the pregnant women, there was no beneficial effect of β-carotene supplementation on anaemia prevalence, neither during pregnancy nor 6 months post-partum (Chapters 8 and 9).

Although an effect of iron supplementation on vitamin A metabolism has not been described before in humans, there are some indications in animal models that iron status affects mobilisation and utilisation of retinol (19,20). In the present studies, infants receiving iron supplementation, either alone or combined with zinc, had markedly reduced plasma retinol concentrations, and a much higher prevalence of vitamin A deficiency (Chapter 5). At the same time, these infants had higher hepatic stores of vitamin A, as shown by the decreased MRDR. This suggests that iron supplementation causes a redistribution of vitamin A from plasma to the liver. As iron supplementation can cause plasma retinol concentrations to fall below the cut-off concentration for vitamin A deficiency, iron supplementation in infants should not be given without measures to improve vitamin A status also. This is especially important in populations were vitamin A status is already marginal. To fully elucidate the underlying mechanisms of the observed redistribution of retinol, animal models are more suitable.

Micronutrients during pregnancy and lactation.

Micronutrient deficiency during pregnancy is associated with increased maternal mortality, increased incidence of complications during pregnancy and labour, lower birth weight, and increased incidence of neonatal complications (10). In this thesis (Chapter 8) we show that supplementation of pregnant women with β-carotene and zinc, in addition to the standard supplementation regime of iron and folic acid, increases birth weight, although only in boys. Other studies have found beneficial effects of supplementation during pregnancy with only retinol or β-carotene in reducing maternal mortality (11), and with only zinc on pregnancy complications and birth weight (12). However, there is reason for caution, as in the studies described in this thesis supplementation during pregnancy with only zinc in addition to iron and folic acid, increased the prevalence of complications during delivery. In view of the concurrent occurrence of deficiency of several micronutrients, supplementation of only zinc or only β-carotene in addition to iron and folic acid might not address all the micronutrient requirements during pregnancy.

Micronutrient supplementation during pregnancy benefits not only the mother but also the infant. The studies described in this thesis show that maternal micronutrient status is a major determinant of the micronutrient status of the infant. A key connection factor between the nutritional status of mothers and infants is of course breast milk. The micronutrient content of breast milk is affected by the micronutrient status of the mother. In Chapters 2 and 9, it is shown that especially retinol and β-carotene content of the breast milk is affected by the vitamin A status of the mother. Moreover, there is a significant relation between the vitamin A content of breast milk, and the vitamin A status of the infant. As already mentioned above, β-carotene supplementation during pregnancy had to be combined with zinc to be able to increase plasma and breast milk retinol concentrations 6 months post-partum. It is encouraging that supplementation during pregnancy is able to significantly improve vitamin A status even 6 months after the end of supplementation, not only in the mothers but also in their infants. In other studies, supplementation of lactating mothers with either a single high dose of retinol directly post-partum or daily with β-carotene did not improve vitamin A status of the infants at 6 months of age (21). In Nepal, pregnant women were supplemented during pregnancy with either retinol or β-carotene. At 3 months of age, plasma retinol concentrations were increased in the newborns, but the effect of retinol supplementation was greater than that of β-carotene (22). In the studies described in this thesis, the effect of β-carotene supplementation during pregnancy was greatly enhanced by the addition of zinc.

Micronutrients and growth.

Micronutrient deficiency has been implicated in the growth impairment during infancy that is seen in many developing countries (6). Especially zinc deficiency has recently been the focus of much interest. Zinc supplementation has been shown to improve growth performance in several studies (23,24). In the studies described in this thesis, zinc deficiency was not related to growth impairment (Chapter 2). Interestingly, there was an association between vitamin A status and growth impairment (Chapter 2). Moreover, zinc supplementation did not improve growth performance (Chapter 4). This is surprising as the prevalence of iron and zinc deficiency are high in these

infants, and iron and zinc supplementation were very effective in decreasing these deficiencies. Apparently, additional factors are involved in the growth impairment in these infants. A recent meta-analysis showed that the effects of supplementation with zinc on growth are small, and most pronounced in stunted infants (25). The studies described in this thesis aimed at preventing stunting during infancy, and not to improve growth in already stunted infants. Therefore, supplementation was started at an age before stunting is prevalent.

Supplementation with micronutrients during pregnancy was not effective in preventing growth impairment in newborn infants during the first 6 months of life, even though birth weight of boys was increased after supplementation during pregnancy with β-carotene and zinc (Chapter 8 and 9). Birth weight however, remained the most important predictor of height-for-age and weight-for-age Z scores at 6 months of age.

Micronutrients and immune function.

Micronutrients are essential for optimal functioning of the immune system. Moreover, micronutrients can modulate the immune response (26). In the studies described in this thesis, micronutrient status distinctly affected immune function in infants. Vitamin A deficiency markedly decreased the ability to produce the type-1 cytokine interferon-γ in whole blood after stimulation (Chapter 3). Intriguingly, circulating neopterin concentrations were higher in vitamin A deficient infants, suggesting increased *in vivo* macrophage activity, which in turn signifies increased stimulation by interferon-γ. Zinc deficiency was accompanied by significantly reduced white blood cell numbers, as well as significantly reduced *ex vivo* production of the type-2 cytokine interleukin-6 (Chapter 3).

Supplementation with iron resulted in higher *ex vivo* interferon-γ production, and lower *ex vivo* interleukin-6 production (Chapter 7). The higher circulating neopterin concentrations found in the infants who received iron supplementation are consistent with the *ex vivo* findings. Supplementation with either β-carotene or zinc seemed to have an opposite effect on immune function (Chapter 7). Overall, these studies show that iron, vitamin A and zinc have marked albeit divergent effects on the immune function of infants. The implications for immunocompetence are less clear. Deficiencies of vitamin A and zinc are known to increase the risk of morbidity and mortality (27,28). An impaired ability to produce pro-inflammatory cytokines in response to infection in infants with deficiency of various micronutrients, as found in the studies described in this thesis, might underlie the increased risk of morbidity and mortality from infectious diseases (29-32). Supplementation with vitamin A has been shown to reduce mortality and morbidity in children (4), and zinc supplementation has been shown to reduce the prevalence and severity of infections, especially in diarrheal disease, respiratory infections and malaria (23,33,34). Iron supplementation is more controversial in this context, and positive as well as negative effects of iron supplementation on immune function are reported (35).

It is important to realise that supplementation of different micronutrients can have opposite immunomodulatory effects. Effects of single micronutrient supplementation can be counteracted by the immunomodulatory effects of deficiency

other micronutrients. Furthermore, single micronutrient supplementation can also lead to unbalanced enhancement or depression of immune response. Therefore, supplementation of more than one micronutrient seems preferable to optimally improve immune function.

CONCLUSIONS AND IMPLICATIONS

From the studies described in this thesis, several conclusions can be drawn with implications for programmes and policy. One conclusion that is recurrent in all the studies described in this thesis, and that warrants to be highlighted, is that concurrent deficiency of several micronutrients are the norm rather than the exception, and that supplementation with more than one micronutrient is preferable and more effective. In populations where micronutrient supplementation is considered, often nutriture of more than one micronutrient is inadequate. The effectiveness of supplementation with only one micronutrient, when multiple deficiencies exist, is considerably reduced as utilisation will not be optimal, and health benefits will fall short of expectations as the other deficiencies are not addressed. Furthermore, single micronutrient supplementation can inadvertently impair absorption, utilisation or metabolism of other micronutrients, especially when nutriture of these micronutrients is already marginal.

Until recently, research efforts were primarily focussed on single micronutrients. Furthermore, the current strategy of most nutrition intervention programmes is to target only one micronutrient deficiency at the time. Iron supplementation for pregnant women, and vitamin A capsule distribution programmes for children are clear examples. When the effort is made to implement a supplementation programme, it would take little extra cost or effort to supply a more complete mixture of micronutrients. The effectiveness of supplementation will be increased in an additive as well as in a synergistic way, and overall health benefits will be much greater.

From the results of the studies described in this thesis, we conclude that supplementation of pregnant women, not only with iron and folic acid, but also with β-carotene and zinc, seems clearly more beneficial than supplementation with iron and folic acid alone, and this would be the easiest change to implement. Furthermore, when considering large scale supplementation in infants, single supplementation of iron would not address concurrent zinc or vitamin A deficiencies, so that a combined supplementation approach is required. However, a significant improvement of growth performance might not be achieved with the combined supplementation of these three micronutrients, as other factors seem to be involved in the growth impairment in these infants also.

RECOMMENDATIONS FOR FUTURE RESEARCH

Interactions between micronutrients are very important, not only in the etiology of micronutrient deficiency, but also during supplementation and in utilisation in metabolic processes. Although many interactions between micronutrients have been described, exact mechanisms are often not yet elucidated. Most often animal models are needed to study metabolic interactions in detail. However, careful and in depth study of deficiencies of micronutrients and the effects of supplementation in human subjects can also give insight in underlying mechanisms and the relative importance of interactions between micronutrients. More research is needed to identify the factors involved in the growth impairment during infancy that is seen in many developing countries. Optimal growth in infants and children is an important health goal, as impaired growth is associated with poor health, increased risk of morbidity and mortality and delayed psycho-motor development.

The important role of the effects of micronutrients in immune function is only recently being recognized. However, immunology is a fast developing field, and many aspects of immune function are still not completely understood, making it even more difficult to investigate exact mechanisms by which micronutrients affect immune function. Elucidating the immunomodulatory effects of micronutrients is very important when considering supplementation of micronutrients, to achieve optimal immunocompetence, and thus optimal reduction of morbidity and mortality.

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SUMMARY

The research described in this thesis was concerned with vitamin A, iron and zinc deficiency in pregnant and lactating women and in infants. The effects of supplementation with β-carotene, iron and zinc on micronutrient status, growth, pregnancy outcome and immune function, and interactions between micronutrients were investigated.

Deficiencies of vitamin A, iron and zinc are prevalent worldwide. Vitamin A deficiency leads to increased risk of morbidity and mortality, and vitamin A supplementation can reduce child mortality by 20-30%, regardless of the prevalence of xerophthalmia in the population. In developing countries, pro-vitamin A carotenoids, especially β-carotene, from plant foods is the most important source of vitamin A, but bioavailability and conversion are highly variable, and less than often assumed. Iron deficiency is the most common micronutrient deficiency globally, affecting over 50% of the women in developing countries. Iron deficiency leads to anaemia, but in children also to delayed psychomotor development and impaired learning, and possibly affects immunocompetence. Diets rich in phytate and low in animal products result in a low availability of iron, and predisposes to iron deficiency.
Zinc availability is impaired by the same factors as iron, and hence zinc deficiency is presumed to be very widespread also. Zinc status however, is difficult to assess due to homeostasis, and the absence of real body stores. Zinc deficiency causes especially growth impairment and decreased immunocompetence, although probably many metabolic functions are affected.

In Indonesia, both vitamin A deficiency and iron deficiency are major health problems, affecting especially pregnant and lactating women, and infants. Zinc deficiency is also thought to be important, and has been implicated in the growth impairment commonly seen in infants in Indonesia. Also, maternal and infant mortality remain high, despite significant improvements in vaccination coverage and access to primary health care. There are several programmes in Indonesia to combat vitamin A and iron deficiency. For example, there is a national iron and folic acid supplementation programme for pregnant women, and there is a programme for intermittent high dose vitamin A supplementation with capsules for children. The vitamin A supplementation programme has successfully decreased the prevalence of xerophthalmia, but marginal vitamin A deficiency remains a problem. Also, because of possible toxicity, young infants and pregnant women are not included in the vitamin A supplementation programme.

The research described in this thesis comprises three studies. The objective of the first study was to assess the prevalence of deficiency of vitamin A, iron and zinc in lactating mothers and their infants as well as the interrelationships of these deficiencies with each other, and with growth impairment and immune function. In the second study, the effects of supplementation of infants with β-carotene, iron and zinc on micronutrient status and deficiency, and on growth and immune function were investigated. In the third study, the effects of supplementation of pregnant women with β-carotene and zinc in addition to iron and folic acid on pregnancy outcome,

micronutrient status of mothers and newborns, and growth of the newborn during the first 6 months were investigated.

The main findings of the research described in this thesis are summarized in relation to several topics important for mother and child health. An important finding in all three studies is not only that deficiency of vitamin A, iron and zinc are prevalent in infants and mothers, but also that these deficiencies are likely to occur concomitantly. Furthermore, micronutrient status of the mother is strongly related to that of her infant, and breast milk is a key connecting factor between mother and infant for vitamin A status.

Supplementation of infants with iron and zinc was effective in reducing the prevalence of anaemia and deficiencies of iron and zinc. Although supplementation of iron combined with zinc reduced the efficacy of iron supplementation, the combined supplement still effectively reduced the prevalence of anaemia and iron deficiency, and in addition reduced zinc deficiency. This shows that although zinc partially inhibits iron uptake, zinc is still a valuable addition to iron supplementation. Supplementation of women during pregnancy with β-carotene combined with zinc improved vitamin A status of both mother and newborn 6 months post-partum, whereas supplementation with β-carotene alone had no effect. This indicates that zinc plays an important role in the conversion of β-carotene to retinol. Therefore zinc is necessary to improve vitamin A status with β-carotene supplementation in pregnant women. The synergistic effect of zinc on β-carotene supplementation is not apparent in infants, but infants may have a reduced capacity to metabolise or store β-carotene. In contrast, iron supplementation was found to have an antagonistic effect on vitamin A status of infants, resulting in lower plasma retinol concentrations, and higher prevalence of vitamin A deficiency in the iron supplemented infants. Concomitantly hepatic stores of vitamin A were increased, suggesting that iron supplementation causes a redistribution of vitamin A from the plasma to the liver. Hence, iron supplementation in infants should not be given without measures to improve vitamin A status also.

Supplementation of pregnant women with β-carotene and zinc improved birth weight, but only in boys. However, supplementation with only zinc in addition to iron and folic acid, increased the prevalence of complications during delivery. In view of the concurrent occurrence of deficiency of various micronutrients, supplementation of only zinc or only β-carotene, in addition to iron and folic acid, may not address all micronutrient requirements during pregnancy.

Although zinc deficiency has been implicated in the growth impairment commonly seen in infants in developing countries, supplementation of infants with zinc did not improve growth performance. Also, supplementation of women during pregnancy with zinc did not improve growth of the infant during the first 6 months of life. Apparently, additional factors are involved in the growth impairment of these infants.

Various micronutrients have profound, albeit different effects on immune function. Infants with vitamin A deficiency had lower *ex vivo* type-1 cytokine response, but higher *in vivo* macrophage activity. Zinc deficiency was accompanied by

reduced white blood cell numbers, as well as lower *ex vivo* type-2 cytokine production. Supplementation with iron resulted in higher type-1, and lower type-2 cytokine production, whilst supplementation with β-carotene and zinc seem to have opposite effects on immune function. Deficiency of various micronutrients can impair the ability to produce pro-inflammatory cytokines in response to infection. Supplementation of different micronutrients can have opposing immunomodulatory effects, but implications for immunocompetence are less clear.

Concluding, the studies described in this thesis clearly and repeatedly show that concomitant deficiencies of various micronutrients are the norm rather than the exception. Supplementation with single micronutrients is not optimal. The expected effectiveness of supplementation with one micronutrient will not be achieved if the utilisation of the micronutrient is impaired by deficiency of another micronutrient. Health benefits of supplementation will also fall short of expectations as long as deficiencies of other micronutrients are not addressed. Therefore supplementation with more than one micronutrient is recommended, not only for infants but also for pregnant women.

SAMENVATTING

Het onderzoek dat in dit proefschrift beschreven wordt, was gericht op het vóórkomen en de effecten van voedingstekorten van vitamine A, ijzer en zink in zwangere en lacterende vrouwen en in jonge kinderen. Ook werd het effect van het suppleren met β-caroteen, ijzer en zink op micronutriëntenstatus, groei, zwangerschapsuitkomst en immuunfunctie onderzocht, alsmede interacties tussen de verschillende micronutriënten.

Wereldwijd komen deficiënties van vitamine A, ijzer en zink vaak voor. Een tekort aan vitamine A verhoogt het morbiditeits- en mortaliteitsrisico, en suppletie van kinderen met vitamine A kan de kindersterfte verminderen met 20 tot 30%, ongeacht de prevalentie van xerophthalmie in de bevolking. In ontwikkelingslanden is β-caroteen, een provitamine A die zich met name in plantaardig voedsel bevindt, de belangrijkste bron van vitamine A. De opname en omzetting van β-caroteen zijn echter hoogst variabel, en lager dan tot voor kort werd aangenomen. Wereldwijd is een ijzerdeficiëntie het meest prevalente voedingstekort, en meer dan 50% van de vrouwen in ontwikkelingslanden heeft een ijzertekort. Een tekort aan ijzer leidt tot bloedarmoede, maar in kinderen kan het ook leiden tot een vertraging van de psychomotore ontwikkeling, een verminderd leervermogen en tot een verminderde afweer tegen infekties. De opname van ijzer uit een dieet dat rijk is aan fytaten, en weinig dierlijke produkten bevat, is laag. Zo'n dieet is gebruikelijk in ontwikkelingslanden: hierdoor wordt de kans op een tekort aan ijzer verhoogt. De opname van zink wordt door dezelfde factoren vermindert als de opname van ijzer. Het is dus waarschijnlijk dat een tekort aan zink ook erg algemeen is. Het meten van de zinkstatus is echter moeilijk door de strikte homeostase en door de afwezigheid van echte lichaamsvoorraden. Duidelijke gevolgen van een zinktekort zijn een verminderde groei en een verminderde afweer tegen infekties, maar waarschijnlijk zijn vele metabole funkties aangedaan.

In Indonesië is een tekort aan zowel vitamine A als ijzer een belangrijk gezondheidsprobleem, met name voor zwangere en lacterende vrouwen en voor jonge kinderen. Waarschijnlijk is een tekort aan zink ook een belangrijk probleem en dit zou een oorzaak kunnen zijn van de groeivertraging zoals die vaak wordt waargenomen bij kinderen in Indonesië. Verder is de maternale sterfte en de kindersterfte nog steeds hoog, ondanks het feit dat er belangrijke vooruitgang is geboekt in de vaccinatiedekkingsgraad en de bereikbaarheid van de 'primary health care' centra. Er zijn verschillende programma's in Indonesië gericht op de preventie van tekorten van vitamine A en ijzer. Zo is er een nationaal programma van ijzer- en foliumzuursuppletie voor zwangere vrouwen en is er een programma waarin met tussenpozen aan kinderen kapsules met een hoge dosis vitamine A wordt verstrekt. De suppletie met vitamine A is erg succesvol geweest in het terugdringen van xerophthalmie, maar het vóórkomen van marginale tekorten aan vitamine A blijft een probleem. Hele jonge kinderen (minder dan 6 maanden oud) en zwangere vrouwen zijn echter uitgesloten van het vitamine A-suppletieprogramma, omdat hogere doses vitamine A voor hun toxisch of teratogeen kunnen zijn.

In dit proefschrift worden 3 verschillende studies beschreven. Het doel van de eerste studie was het vaststellen van de prevalentie van tekorten aan vitamine A, ijzer en zink in lacterende moeders en hun kinderen. Ook werden de onderlinge verbanden tussen deze tekorten en de relatie met groei en immuniteit onderzocht. In de tweede studie werden de effecten onderzocht van suppletie van kinderen met β-caroteen, ijzer en zink op de voedingsstatus en de prevalentie van tekorten, alsmede op de groei en de immuniteit. In het derde onderzoek werden de effecten van suppletie van zwangere vrouwen met β-caroteen en zink (naast de standaardsuppletie met ijzer en foliumzuur) onderzocht op de zwangerschapsuitkomst, de voedingsstatus van moeder en kind en de groei van het kind gedurende de eerste 6 maanden onderzocht.

De belangrijkste bevindingen van het onderzoek zullen hieronder thematisch worden samengevat, aan de hand van onderwerpen die van belang zijn voor de gezondheid van moeder en kind. Een belangrijke bevinding in alle drie de studies is dat tekorten aan vitamine A, ijzer en zink niet alleen vaak vóórkomen, maar dat ze ook vaak tegelijkertijd vóórkomen. Verder is de micronutriëntenstatus van de moeder sterk gerelateerd aan die van haar kind, en is moedermelk een belangrijke verbindende faktor tussen de vitamine A-status van moeder en kind.

Suppletie met ijzer en zink was effectief in het verminderen van het vóórkomen van bloedarmoede en tekorten van ijzer en zink. Hoewel de werkzaamheid van suppletie wordt verminderd door het combineren van ijzersuppletie met zinksuppletie, was het gecombineerd suppleren nog steeds effectief in het verminderen van de prevalentie van bloedarmoede en ijzertekort, alsmede van zinktekort. Dit toont aan dat hoewel zink de opname van ijzer gedeeltelijk remt, zink toch een waardevolle toevoeging is aan ijzersupplementen. Het suppleren van vrouwen tijdens de zwangerschap met de combinatie van β-caroteen en zink verbeterde de vitamine A-status van zowel moeders als hun kinderen 6 maanden post-partum, terwijl suppletie met alleen β-caroteen geen effect had. Dit wijst op een belangrijke rol van zink in de omzetting van β-caroteen naar retinol. Zink is dan ook noodzakelijk indien men de vitamine A-status van zwangere vrouwen wil verbeteren met behulp van β-caroteensuppletie. Dit synergistische effect van zink op de effectiviteit van β-caroteensuppletie is niet aanwezig in de gesuppleerde kinderen, maar het kan zijn dat kinderen een verminderde capaciteit hebben om β-caroteen te metaboliseren of op te slaan. IJzersuppletie daarentegen bleek een antagonistisch effect op de vitamine A-status van kinderen te hebben, resulterend in een hogere prevalentie van vitamine A-tekort in kinderen die ijzersupplementen kregen. Deze kinderen hadden echter tegelijkertijd ook grotere levervoorraden van vitamine A, wat er op wijst dat ijzersuppletie leidt tot een herverdeling van vitamine A vanuit het bloed naar de lever. Daarom zou ijzersuppletie gecombineerd moeten worden met maatregelen om tegelijkertijd de vitamine A-status te verbeteren.

Het suppleren van zwangere vrouwen met β-caroteen en zink gaf een verhoging van het geboortegewicht van jongens, maar niet van meisjes. Daarentegen leidde het suppleren van zwangere vrouwen met alleen maar zink, naast de standaard ijzer- en foliumzuursuppletie, tot een toename van het aantal complicaties tijdens de bevalling. Gelet op het vaak gelijktijdig vóórkomen van meerdere voedingstekorten, is het goed mogelijk dat het suppleren met alleen maar β-caroteen of alleen maar zink, naast de

standaard ijzer- en foliumzuursuppletie, niet voldoende is om te voorzien in alle micronutriënten waar tijdens de zwangerschap behoefte aan is.

Alhoewel een tekort aan zink is genoemd als oorzaak van de verminderde groei van kinderen in ontwikkelingslanden, hielp suppletie van kinderen met zink niet in het verbeteren van de groei. Ook kinderen van moeders die tijdens de zwangerschap met zink gesuppleerd waren, groeiden niet beter dan andere kinderen. Blijkbaar zijn er andere faktoren die leiden tot de verminderde groei van deze kinderen.

Verschillende micronutriënten hebben een duidelijk, maar verschillend effect op het immuunsysteem. Kinderen met een vitamine A-tekort hadden een lagere *ex vivo* produktie van type-1 cytokines. Kinderen met een zinktekort hadden minder witte bloedlichaampjes en ook een lagere *ex vivo* produktie van type-2 cytokines. Het suppleren van kinderen met ijzer leidde tot een hogere produktie van type-1 cytokines, en een lagere produktie van type-2 cytokines, terwijl suppletie met β-caroteen en zink het omgekeerde effect lijken te hebben. Tekorten aan micronutriënten kan het vermogen van het immuunsysteem om pro-inflammatoire cytokines te produceren in reactie op een infectie verminderen. Suppletie van verschillende micronutriënten heeft tegenovergestelde effecten op het immuunsysteem, maar de implicaties hiervan voor de afweer tegen infektie zijn minder duidelijk.

Concluderend heeft het onderzoek zoals beschreven in dit proefschrift duidelijk en bij herhaling aangetoond dat het gelijktijdig vóórkomen van tekorten aan meerdere micronutriënten eerder de regel dan de uitzondering is. Het suppleren met slechts 1 micronutriënt is niet optimaal. Het verwachtte effect van suppletie van een micronutriënt zal niet bereikt kunnen worden als opname en gebruik van het micronutriënt verminderd worden door tekorten aan andere micronutriënten. Ook zullen verbeteringen in de (volks-)gezondheid achterblijven bij de verwachtingen zolang niets aan de andere tekorten gedaan wordt. Het suppleren met meerdere micronutriënten verdient dus de voorkeur, zowel wat betreft kinderen als zwangere vrouwen.

RINGKASAN

Penelitian yang digambarkan dalam tesis ini titik fokusnya adalah defisiensi vitamin A, zat besi, dan seng pada wanita hamil dan menyusui serta pada bayi. Yang diteliti adalah pengaruh suplementasi beta karotin, zat besi, dan seng terhadap status mikronutrien, pertumbuhan, hasil kehamilan, dan interaksi antar mikronutrien.

Defisiensi vitamin A, zat besi, dan seng merupakan masalah kesehatan dunia. Defisiensi vitamin A dapat mengakibatkan, meningkatnya risiko morbiditas dan mortalitas, dan penambahan vitamin A dapat mengurangi kematian anak sampai 20-30%, tanpa memperhatikan besarnya prevalensi xeroftalmia di masyarakat. Di negara-negara berkembang, karotinoid pro-vitamin A – terutama betakarotin – dari tanaman pangan merupakan sumber utama vitamin A, tetapi bioavailabilitas dan konversinya sangat bervariasi dan kurang dari yang biasa diasumsikan. Defisiensi zat besi merupakan masalah defisiensi mikronutrien global yang paling umum, menjangkiti lebih 50% wanita di negara-negara berkembang. Defisiensi zat besi dapat menyebabkan anemia, sedangkan pada anak juga dapat menyebabkan lambatnya perkembanguan psikomotorik dan gangguan proses belajar, serta adanya kemungkinan dampak pada *immunocompetence*. Makanan yang kaya fitat dan rendah hewani menyebabkan kurang tersedianya zat besi dan cenderung mengakibatkan defisiensi zat besi. Ketersediaan seng juga dipengaruhi oleh faktor-faktor yang sama dengan zat besi, sehingga defisiensi seng juga diyakini tersebar luas. Namun, status seng sulit ditentukan karena sifatnya yang *homeostasis* dan tidak tersimpan dalam bentuk nyata. Defisiensi seng menyebabkan terutama gangguan pertumbuhan dan menurunnya daya tahan tubuh, meskipun beberapa fungsi metabolisme mungkin terganggu.

Di Indonesia, baik defisiensi vitamin A maupun zat besi merupakan masalah kesehatan yang utama, yang mempengaruhi wanita hamil dan menyusui, serta bayi. Defisiensi seng juga merupakan masalah penting, yang telah menyebabkan gangguan pertumbuhan pada anak-anak Indonesia. Demikian juga, tingkat kematian ibu dan anak tetap tinggi, meskipun telah ada perbaikan yang signifikan dalam cakupan vaksinasi dan akses terhadap sarana kesehatan. Ada beberapa program di Indonesia yang bertujuan menanggulangi defisiensi vitamin A dan zat besi. Contohnya program nasional suplementasi zat besi dan asam folat bagi wanita hamil, program suplementasi vitamin A dosis tinggi dalam bentuk kapsul pada anak-anak. Program suplementasi vitamin A telah berhasil menurunkan prevalensi xeroftalmia, tetapi defisiensi vitamin A marginal masih tetap merupakan masalah. Dimikian juga, karena kemungkinan keracunan, bayi-bayi muda dan wanita hamil tidak diikutsertakan dalam program suplementasi vitamin A.

Penelitian yang digambarkan dalam tesis ini terdiri dari tiga kajian. Tajuan dari kajian pertama adalah mengukur prevalensi defisiensi vitamin A, zat besi, dan seng pada wanita menyusui dan bayi mereka; hubungan timbal balik antara masing-masing defisiensi, dan dengan gangguan pertumbuhan serta fungsi kekebalan. Dalam kajian kedua diteliti pengaruh suplementasi betakarotin, zat besi, dan seng pada bayi terhadap defisiensi dan status mikronutrien, pertumbuhan, dan fungsi kekebalan. Dalam kajian ketiga, diteliti pengaruh suplementasi betakarotin dan seng pada ibu hamil sebagai tambahan pada zat besi dan asam folat terhadap hasil kehamilan, status mikronutrien

ibu dan bayi yang baru dilahirkan, serta pertumbuhan bayi selama enam bulan pertama setelah kelahiran.

Temuan-temuan penting dari riset yang dijarbarkan dalam tesis ini dirangkum sesuai dengan topik-topik penting bagi kesehatan ibu dan anak. Temuan penting dari ketiga kajian tersebut tidak hanya bahwa defisiensi vitamin A, zat besi , dan seng prevalen pada bayi dan ibu, tetapi juga defisiensi tersebut terjadi secara bersamaan. Lebih jauh, status mikronutrien ibu mempunyai hubungan yang kuat dengan status bayinya, dan air susu ibu merupakan kunci faktor penghubung antara ibu dan bayi kuhususnya untuk status vitamin A.

Suplementasi zat besi dan seng pada bayi akan efektif menuruukan prevalensi anemia, defisiensi zat besi dan seng. Meskipun suplementasi zat besi yang dikombinasikan dengan seng mengurangi efikasi suplementasi zat besi, kombinasi suplemen tersebut tetap efektif menurunkan prevalensi anemia dan defisiensi zat besi, dan selanjutnya menurunkan defisiensi seng. Hal ini menunjukkan bahwa meskipun seng secara terpisah menghambat zat besi, seng tetap merupakan tambahan penting bagi suplementasi zat besi. Suplementasi betakarotin selama kehamilan yang dikombinasikan dengan seng meningkatkan status vitamin A baik bagi ibu maupun bagi bayinya sampai enam bulan setelah kelahiran, sementara suplementasi hanya dengan betakarotin tidak mempunyai efek. Ini menunjukkan bahwa seng memainkan peran penting dalam konversi betakarotin menjadi retinol. Dengan demikian seng diperlukan untuk meningkatkan status vitamin A pada wanita hamil bersama dengan suplementasi betakarotin. Efek sinergis seng pada suplementasi betakarotin tidak nampak pada bayi, mungkin karena bayi memiliki kemampuan yang rendah dalam memetabolisir atau menyimpan betakarotin. Sebaliknya, suplementasi zat besi memiliki efek antagonis terhadap status vitamin A bayi, yaitu mengakibatkan menurunnya konsentrasi plasma retinol dan meningkatkan prevalensi defisiensi vitamin A pada bayi yang disuplementasi zat besi. Secara bersamaan, simpanan vitamin A dalam hati meningkat, yang berati bahwa penambahan zat besi menyebabkan redistribusi vitamin A dari plasma ke hati. Dengan demikian, suplementasi zat besi pada bayi sebaiknya tidak diberikan tanpa langkah perbaikan status vitamin A juga.

Suplementasi betakarotin dan seng pada wanita hamil dapat meningkatkan berat badan lahir bayi, tetapi hanya pada payi laki-laki. Meskipun demikian, suplementasi seng saja sebagai tambahan pada zat besi dan asam folat meningkatkan angka komplikasi kelahiran. Dilihat dari defisiensi macam-macam mikronutrien yang terjadi secara simultan, suplementasi hanya seng atau hanya betakarotin saja sebagai tambahan pada zat besi dan asam folat, mungkin tidak dapat memenuhi semua mikronutrien yang dibutuhkan selama kehamilan.

Meskipun defisiensi seng berdampak pada gangguan pertumbuhan bayi yang umum terdapat di negara berkembang, suplementasi seng pada bayi tidak memperbaiki keadaan pertumbuhan tersebut. Demikian juga, suplementasi seng pada wanita selama kehamilan tidak memperbaiki pertumbuhan bayi selama usia 6 bulan pertama. Nampaknya ada faktor-faktor lain yang juga berpengaruh dalam gangguan pertumbuhan bayi-bayi tersebut.

Berbagai mikronutrien memiliki efek yang besar dan berbeda-beda pada fungsi kekebalan. Bayi yang mengalami defisiensi vitamin A memiliki respon *cytokine tipe-1 ex vivo* yang lebih rendah, tetapi memiliki aktifitas makrofag *in vivo* yang lebih tinggi. Defisiensi seng disertai oleh menurunnya jumlah sel darah putih dan lebih rendahnya produksi *cytokine tipe-2 ex vivo*. Suplementasi dengan zat besi mengakibatkan meningkatnya produksi *cytokine tipe-1*, dan menurunnya *cytokine tipe-2*, sementara suplementasi dengan betakarotin dan seng tampak memiliki efek yang berlawanan pada fungsi kekebalan. Defisiensi berbagai macam mikronutrien dapat menghambat kemampuan produksi *cytokine pro-inflamasi* dalam merespon infeksi. Suplementasi mikronutrien yang berbeda-beda dapa menyebabkan efek *immunomodulatory* yang berlawanan, tetapi dampak terhadap *immunocompetence* kurang terlihat jelas.

Disimpulkan, kajian-kajian yang digambarkan dalam tesis ini yang secara jelas menunjukkan bahwa adanya defisiensi berbagai mikronutrien yang terjadi bersamaan lebih merupakan norma daripada eksepsi. Suplementasi dengan mikronutrien tunggal pada kasus yang disertai defisiensi mikronutrien lain, kurang efektif karena utilisasinya menjadi tidak optimal, dan keuntungan kesehatan (*health benefits*) tidak akan seperti yang diharapkan selama defisiensi lainnya tidak teratasi. Dengan demikian suplementasi lebih dari satu mikronutrien adalah lebih baik dan lebih efektif, tidak hanya bayi tetapi juga bagi wanita hamil.

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Marjoleine Amma Dijkhuizen was born on Saturday, June 1, 1968 in Tema, Ghana. She completed her secondary school at the Gooi's Lyceum, Bussum in 1987, and in the same year she enrolled in the faculty of Medicine at the University of Amsterdam. In 1990, after completing the third year of Medicine, she worked for one year at the Tropical Metabolism Research Unit (TMRU), University of the West-Indies, Jamaica on a joint research project with Frank Wieringa, on the pharmacokinetics of gentamicin in malnourished children, and on the immune response of malnourished children. She also took up the study of Medical Biology at the University of Amsterdam, and worked on the cloning of the pyrroloquinoline quinone gene in *Escherichia coli*, as a final year student. She joined the TEMPUS course on the Molecular Basis of Metabolic Diseases in Amsterdam and Prague in 1993. Her MSc. thesis on "The Relationship Between Malaria and Nutrition" won the NVVL price for the best MSc. thesis in 1994. In June 1993 she received her MSc. degree in Medicine, and in August 1993 her MSc. degree in Medical Biology. She graduated as a medical doctor in January 1996. During her last internship she worked four months at Kaoma Hospital, Zambia as junior doctor. She started with the Ph.D. research project described in this thesis in May 1996.

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PUBLICATIONS

Scientific publications related to this thesis.

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