Zinc and infectious disease – studies of mice and men

Doctoral thesis by
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**Appendix:** Quality Assurance of the Clinical Trial in Nepal
“If we could give every individual the right amount of nourishment and exercise, not too little and not too much, we would have found the safest way to health.”

Hippocrates 460 - 377 B.C
Acknowledgements

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List of papers

The thesis is based on the following papers:


**Abbreviations**

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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<td>ALRI</td>
<td>acute lower respiratory infection</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
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<td>ARR</td>
<td>absolute risk reduction</td>
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<td>CI</td>
<td>confidence interval</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<td>GEE</td>
<td>generalized estimation equations</td>
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<td>GPS</td>
<td>global positioning system</td>
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<tr>
<td>IMCI</td>
<td>integrated management of childhood illness</td>
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<td>IU</td>
<td>international units</td>
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<tr>
<td>LCI</td>
<td>lower chest indrawing</td>
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<tr>
<td>NNT</td>
<td>numbers needed to treat</td>
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<tr>
<td>NRS</td>
<td>Nepalese rupees</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
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<td>ORS</td>
<td>oral rehydration solution</td>
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<td>PspA</td>
<td>pneumococcal surface protein A</td>
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<tr>
<td>RDA</td>
<td>recommended daily allowances</td>
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<td>RR</td>
<td>relative risk</td>
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<td>SCD</td>
<td>sickle cell disease</td>
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<td>SGA</td>
<td>small for gestational age</td>
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<td>WHO</td>
<td>World Health Organization</td>
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Summary

Zinc is an essential trace element important for almost all biological systems. Zinc nutriture is likely to be suboptimal in many children of developing countries and may contribute to their impaired growth, increased susceptibility to infections and possibly to the high mortality. A large proportion of childhood deaths in developing countries are caused by diarrhea and pneumonia. Impaired zinc nutriture seems to play a role in the increased incidence and severity of these infections.

As a basis for this thesis work, we undertook two randomized placebo controlled trials and two animal experiments to study the effect of zinc in common childhood illnesses. In Nepal, 1,792 children were randomized to receive zinc or placebo. This trial was designed to assess: the efficacy of oral zinc given during acute diarrhea, whether zinc was as effective when given by the caretaker as when given by a trained field worker, whether the effect of zinc was dependent on concomitant vitamin A administration, whether zinc was associated with any adverse effects, and whether there was any beneficial effect of zinc administration beyond the enrollment episode. Furthermore, a blood sample drawn at enrollment enabled us to measure the associations between the plasma zinc concentration and markers of infection severity. In another trial in 2,482 Indian children, we assessed whether daily zinc administration for 4 months reduced the incidence and risk of acute lower respiratory tract infections (ALRI) and pneumonia. Two animal experiments were carried out to assess whether zinc depleted mice had reduced immune responses to pneumococcal antigens, whether they had an increased infection severity following mucosal pneumococcal challenge, and whether immunization of mice with pneumococcal surface protein A (PspA) before challenge
could reduce the infection severity and the inflammation-associated depletion of plasma and bone zinc.

Nepalese children that were given zinc during acute diarrhea had a substantial reduced risk of prolonged and persistent diarrhea. This study also demonstrated that the caretakers were excellent providers of zinc and that the effect of zinc was not dependent on vitamin A administration. Zinc administration, however, was not associated with a reduced incidence of ALRI, pneumonia or diarrhea during the month after cessation of the enrollment episode. The plasma zinc concentration was lower in children with elevated axillary temperature, increased plasma C-reactive protein (CRP) concentrations and in those with dysentery upon enrollment. In the trial on routine zinc administration in Indian children, the proportion of children who had ALRI was not different in the two experimental groups. Zinc supplementation, however, substantially reduced the risk of clinical pneumonia. Vomiting was more common in children receiving zinc in both clinical trials.

In the first animal experiment, there was no measurable effect of zinc depletion on the anti-pneumococcal polysaccharide T cell independent IgM response. However, in the second experiment, mice on the zinc deficient diet showed substantially reduced T cell dependent immune responses to PspA, more extensive pneumococcal colonization in the nasal mucosa, more severe infections and an increased risk of death. PspA immunization reduced the risk of severe disease; the reduction in severity was reflected in substantially reduced zinc depletion from bones and plasma but not in a measurable reduction in the death risk.
Zinc administration reduced the incidence and risk of clinical pneumonia, which is a serious infection that causes 2-3 million deaths in young children every year. Another 2-3 million children die of diarrhea every year and most of these deaths are associated with prolonged episodes. Thus, a substantial decrease in the incidence of pneumonia with improvement of zinc nutriture and a reduction in the risk of persistent diarrhea with adjunct zinc therapy may contribute to improving child health and survival in developing countries.
Introduction

The first published reports of experiments demonstrating the role of zinc in mammals were undertaken some 70 years ago (1). The importance of zinc nutriture in human health was recognized 30 years later when stunting and delayed sexual maturation in Iranian adolescent males was attributed to zinc deficiency (2-4). In the 1970s, it was discovered that the rare inherited disorder Acrodermatitis enterophatica was caused by impaired zinc absorption and that it could be effectively treated with large doses of oral zinc (5-7). Features of this disease are stunted growth and increased susceptibility to infections such as those causing diarrhea. The impaired growth and high burden of infectious diseases seen in many marginalized populations was then linked to poor zinc nutriture, recognizing the possible public health importance of zinc deficiency (8-10). This initiated extensive research on zinc in human health and several studies have since then described the negative consequences of poor zinc nutriture on human health (11-18).

Zinc nutrition

Foods that are rich in bioavailable zinc are relatively expensive and thereby not available for many. Absorption is dependent on the amount of zinc and its solubility in the intestinal lumen. Phytic acid, which is present in legumes (e.g. beans, peas, lentils and ground nuts) and cereals (e.g. corn and sorghum) reduces the solubility of zinc in the gut and impairs its absorption (19-24). Meat, fish and other animal products are rich in zinc, contain no phytic acid and are accordingly good sources of bioavailable zinc.
Animal products also contain amino acids that promote zinc absorption (19, 25). Furthermore, the demand for zinc is high in individuals with a rapid growth rate, i.e. fetuses, children and adolescents (14). Zinc is excreted in the stools and more zinc is lost during diarrheal illnesses (26). Thus, developing country children, who have a diet with a low content of bioavailable zinc and who experience frequent episodes of diarrhea are at a particularly high risk of zinc deficiency. Indeed, Brown and Wuehler have estimated the percent of the population at risk of low zinc intake in 178 countries using information on the total daily per capita amount of zinc and phytic acid consumption and the zinc requirements (10). According to these calculations, almost half of the world’s population and 95 % of the South Asian population are at risk of low zinc intake.

**Zinc in health and disease**

Zinc is present in most biological systems. It is required for RNA, DNA and protein synthesis, cellular division, differentiation and growth (14, 27). Zinc is important for the configuration and for the catalytic and regulatory action of more than 300 known enzymes in man and it is important for the structure and function of cellular membranes (28, 29). Although it may induce vomiting (10, 30), zinc is not considered to be toxic even in relatively high doses. Excess zinc in the diet is not absorbed and stored in the body for later use, and there is no known disorder that is associated with its accumulation (14).

As zinc is crucial for cellular growth and differentiation (27); cells with a rapid turnover, e.g. those of the respiratory and intestinal epithelium, skin, gonads, and
lymphoid tissues, are particularly sensitive to alterations in available zinc (14). Zinc interacts with several growth factors, particularly growth hormone and insulin-like growth factor-I, being important for their synthesis and modulating their actions (27, 31). It is required to maintain an adequate appetite, taste and smell, which might be affected even in mild zinc deficiency (14, 32).

**Zinc and the immune system.** The effect of zinc on the immune system has been extensively studied in animals and humans as well as *in vivo* and *in vitro* (14, 33, 34). Impaired zinc status affects almost all facets of the immune system and a review on zinc and the resistance to infections by Shankar and Prasad concludes: “Although the effects of zinc deficiency on immunity are profound and ubiquitous, it is remarkable that greater effects on health are not observed in marginally zinc-deficient populations.” (34). The function of the immune system depends on the ability of its cells to proliferate and differentiate, which is impaired in individuals with suboptimal zinc nutriture (27). Moreover, adequate macrophage function, which is important in the initial steps of the immune response, is also highly sensitive to zinc deficiency (35-38). Studies in zinc deficient animals and humans have described considerable reductions in the size of the thymus, the central organ for T cell development (39-44). In fact, impaired T cell dependent immunity with a reduction in the number of T cells in the peripheral lymphoid organs and a reduced CD4/CD8 ratio are of the initial findings during zinc depletion (34). Furthermore, zinc deficiency is associated with a switch from predominantly Th1 to Th2 responsiveness, which is reversed upon zinc administration (45-47).
Resistance to infection is also dependent on adequate mechanical barriers provided by the skin and mucosal surfaces. The integrity of the linings of the gastrointestinal (48, 49) and pulmonary tracts (50) is compromised during zinc deficiency and is likely to contribute to increased susceptibility to infections (14, 34). Zinc is an antioxidant and is able to inhibit lipopolysaccharide and interleukin 1(beta) -induced NO formation (51). Thus, during infections in individuals with a suboptimal zinc nutriture, impairment of the components of the innate and acquired immunity may be aggravated by increased oxidative stress. The number of cells undergoing apoptosis increases in a variety of tissues of zinc deficient animals and humans (34, 48, 52, 53). This may again contribute to the reduced number of lymphocytes and disruption of the epithelial barriers.

The effect of infection on tissue zinc. Studies in humans and animals show that stress, inflammation, and infection reduce the plasma zinc concentration (54-59). The reduction in plasma zinc seems to reflect the severity of the infection (57, 58) and may be observed early during the illness, even before signs of infection, for example fever, appear (60). Organs such as the skin, thymus, bones and the epithelium also become depleted during this process (61). In studies in Pakistan, however, only severe infections such as pneumonia and sepsis were found to be associated with depressed plasma zinc levels in children (62). Moreover, a review by Brown (59) suggests that common, acute infections encountered in community settings may not have a major effect on the plasma zinc concentration. This review also suggests, however, that fever might be a useful marker of whether or not a particular infection affects the plasma zinc concentration.
There is a hypothetical possibility that the redistribution of zinc during infections has a beneficial effect, e.g. in reducing the amount of available zinc for the causative agent, analogous to what has been hypothesized for iron (63, 64) and that zinc deficiency may affect various microorganisms differently (65). Results from clinical trials, however, do not suggest that zinc has a harmful effect when given during infections or when given routinely to prevent infections (12, 13, 18, 66-85).

**Assessment of zinc status**

In the absence of other good markers, plasma and serum zinc concentration remain the most commonly used indicators of zinc status in humans. Plasma and serum zinc, however, may not reflect zinc status well because less than 1% of the total body zinc circulates in the plasma and because the plasma zinc concentration is affected by several conditions. In addition to the above mentioned effect of inflammation, plasma zinc levels also vary with the proximity of the last meal (86, 87), recent exercise (88) and the time of the day (89).

**Interaction with vitamin A**

Several studies have assessed the interaction between vitamin A and zinc. Christian and West who recently reviewed this topic concludes that “Absorption, metabolism, hepatic release, transport, and tissue utilization of vitamin A may depend, in part, on adequate zinc status” (90). Later, studies in Mexico (91) and Bangladesh (92) have shown that administration of zinc to children improves biochemical indicators of vitamin A status. Furthermore, based on a subgroup analysis in a study assessing the effect of zinc and/or
vitamin A administration in night-blind Nepalese women (93), the authors concluded that zinc enhanced the effect of vitamin A in restoring night vision in those with low initial serum zinc concentrations. Zinc deficient individuals may thus face the consequences of secondary vitamin A deficiency in addition to the detrimental effects of impaired zinc nutriture. Vitamin A administration, however, in malnourished children (in whom zinc deficiency may be common) seems to have a large impact in reducing the occurrence of night blindness, anemia, and the severity of infections, as well as mortality without concomitant zinc supplementation (94-99). In a recent vitamin A and zinc supplementation trial in Bangladeshi children, there was a significant interaction between zinc and vitamin A on the delayed effects, i.e. on the incidence and prevalence of diarrhea and pneumonia (100). Thus, a short course of zinc was more efficacious in reducing the subsequent 6-month incidence and prevalence of diarrhea when given with vitamin A. Moreover, the short-term zinc administration was seemingly associated with an increased incidence of pneumonia, which was not present in those given vitamin A along with zinc.

In many of the therapeutic trials showing a beneficial effect of zinc on diarrhea, vitamin A was given to all participants (12, 68, 79, 83, 84). Adequate vitamin A nutriture may be a prerequisite for the absorption and the utilization of zinc. This was demonstrated in an experiment where severely vitamin A deficient chicks had substantially reduced zinc absorption, which was restored following vitamin A administration (101). However, trials on vitamin A and zinc during childhood diarrhea have failed to demonstrate an interaction between these two nutrients (69, 71).
**Malnutrition**

Figures from the WHO indicate that 174 million developing country children under 5 years of age are underweight, and that 230 million are stunted (102). Malnutrition results in poor physical growth, impaired resistance to infections, and perhaps suboptimal cognitive development (103). It is has been estimated that 55% of the 12.2 million deaths among children under 5 years of age is associated with malnutrition (103, 104). Deficiency of multiple micronutrients is more often than not part of malnutrition and it is difficult to estimate the relative importance of the deficiencies of the individual nutrients. For example, zinc deficiency has detrimental effects on growth and immunity, but so does general malnutrition (105). The results from several clinical trials examining the effect of supplementation with various micronutrients, however, indicate that zinc deficiency contributes substantially to growth failure as well as to increased diarrheal and respiratory morbidity in developing country children (12, 13, 16-18, 66, 106).

**Common infectious diseases in children of developing countries**

Globally, most childhood deaths are caused by common infections such as acute lower respiratory infections (ALRI), diarrhea, malaria and measles (104, 107). Treatment of these diseases is feasible but malnutrition, unavailability of and/or inadequate health care services and other factors related to poverty contribute to the high disease burden (103, 104, 108).
Clinical trials of zinc supplementation in children of developing countries

Growth

Several clinical trials have been carried out to assess the effect of zinc on growth; Brown and coworkers have summarized the results of 33 such trials in a recent meta-analysis (16). They found that zinc supplementation resulted in increased height and weight gains, the mean change in SD units height-for-age and weight-for-age gains was 0.35 (95% CI 0.19, 0.51) and 0.31 (0.18, 0.44), respectively. The effect of zinc supplementation was larger among the stunted, which is supported by results from two individual clinical trials that analyzed the effect of zinc among stunted and non-stunted children separately (106, 109).

Diarrhea

Approximately 2-3 million children die of diarrhea every year (110, 111). The mortality from acute dehydrating childhood diarrhea has been declining, mainly as a consequence of the introduction of oral rehydration therapy (112). Other efforts that also may have contributed to the mortality reduction are promotion of breastfeeding, improved supplemental feeding, female education, immunization against measles, and improvements in hygiene and socioeconomic status (112).

Diarrhea may be classified into three syndromes, acute diarrhea, persistent diarrhea and dysentery. Persistent diarrhea is defined as diarrhea of a presumed infectious cause with acute onset that lasts for at least 14 days (113). Persistent diarrhea, where the nutritional
insult is the main danger, is now the leading cause of diarrheal deaths in children of developing countries (113, 114). Reduced host immunity, in HIV-negative children to a large extent caused by malnutrition and micronutrient deficiencies, plays an important role in the development of this syndrome (113). Prevention and correction of malnutrition may accordingly be important measures to reduce the risk and burden of persistent diarrhea.

**Treatment of diarrhea with zinc.** Children with low plasma zinc concentration are more susceptible to diarrhea as compared to those with normal levels (11). During diarrhea, zinc absorption may be impaired and zinc is lost in the stools (115). Zinc deficiency affects the mucosal integrity and epithelial function (14, 116), and zinc administration has been shown to improve the function of the intestinal mucosa (49, 117, 118). Sachdev and coworkers were the first to assess the therapeutic effects of zinc during diarrhea in developing country children (83). They found that children who were given 40 mg of elemental zinc daily had a shorter diarrheal duration and lower stool frequency than the controls. Following this publication, several trials on zinc administration during acute and persistent diarrhea have been conducted and the results from many of these trials have been summarized in a pooled analysis (12). The analysis that included children with acute diarrhea showed that there was an overall and statistically significant reduced risk of continuation of diarrhea on any given day in children receiving zinc (Hazard ratio: 0.85, 95% CI 0.76, 0.95). The risk of having an episode lasting >7 days was reduced by 19% but the effect of zinc on this outcome did not reach statistical significance. Zinc supplementation during acute diarrhea also reduced the stool frequency.
In the trials that enrolled children with persistent diarrhea, therapeutic zinc reduced the overall risk of continuation of diarrhea (Hazard ratio: 0.76, 95% CI 0.63, 0.91) and the odds of treatment failure or death (odds ratio [OR] 0.61, 95% CI 0.26, 1.46). No trials in children with bloody diarrhea, i.e. dysentery, where antibiotic treatment is indicated, were included. After this pooled analysis was published, more studies on the therapeutic use of zinc in diarrhea have been competed (119-122). The results of these studies were presented in a WHO meeting in New Delhi in 2001 and the report from this meeting (123) concludes: “Based on the results of this review, it is concluded that there is now enough evidence demonstrating the efficacy of zinc supplementation on the clinical course of diarrhoea, with regard to the severity and duration of the episode”. However, the meeting also concluded that “effectiveness studies to assess the feasibility, sustainability, and cost-effectiveness of different strategies for delivering zinc supplementation should be undertaken.” The results from paper I (121) and a large cluster-randomized trial in Bangladesh (119) are included in this report. In the latter trial, therapeutic zinc were offered to children with acute diarrhea in half of the clusters. An increased ORS usage of 20%, a reduced antibiotic usage of 60%, and a faster recovery was observed in the clusters where zinc was distributed (123). There were 40 non-injury fatalities in the trial and the risk of such deaths was 51% (95% CI 5%, 75%) lower in the children of the clusters that were offered zinc.

**Prevention of diarrhea with zinc.** Clinical trials in children, usually from relative marginalized strata of developing countries, have shown that routine zinc supplementation reduces the incidence and prevalence of diarrhea (18, 72, 74, 76, 82, 85, 106, 124). A pooled analysis of zinc supplementation trials used published and unpublished data from 10 developing countries to assess the efficacy of daily zinc
administration on diarrheal morbidity (13). This analysis concluded that zinc-supplemented children had an 18% (95% CI 7%, 28%) lower incidence rate and a 25% (95% CI 12%, 37%) lower prevalence of diarrhea. This publication also included a pooled analysis from three trials that used short-term zinc supplementation, i.e. where the children were supplemented with 2-4 recommended daily allowances (RDA) (125) zinc for two weeks and then observed for another 2 to 3 months. The combined pooled effect of these short courses of zinc supplementation on the prevalence (OR 0.66, 95% CI 0.52, 0.83) and incidence rate (OR 0.89, 95% CI 0.62, 1.28) of diarrhea was similar to what was seen in the trials supplementing children with zinc throughout the observation period. Later studies have confirmed this reduction in diarrheal morbidity in routine (18, 74) and in short term zinc supplementation (100), in trials with relatively large sample sizes.

**Respiratory tract infections**

Pneumonia and other ALRIs kill more than two million children every year, most of whom live in developing countries (126-128). The very young, the elderly, and those with conditions that interfere with immunity such as sickle-cell disease and HIV infection are at particularly high risk of severe disease (129). Pneumonia is usually treated successfully with inexpensive antibiotics, the high disease burden in many developing countries may to a large extent be ascribed to unavailability of adequate health care (108) as well as to impaired immunity due to malnutrition as well as specific infections, including HIV infection. According to the WHO’s Integrated management of childhood illnesses (IMCI) program, (none-severe) “pneumonia” is operationally defined to be present in a child with cough or difficult breathing who has elevated respiratory rate and is intended to encompass other ALRI as well as
pneumonia (130). This operational definition of pneumonia has been recommended because of its simplicity and high sensitivity although it has a low specificity. In a child with cough or difficult breathing, the IMCI definition of "severe pneumonia" is based on the presence of lower chest indrawing (LCI), nasal flaring, or any of the danger signs including convulsions, lethargy or unconsciousness, or inability to drink or breastfeed. "Clinical pneumonia" is usually defined by clinicians and some researchers to be pneumonia diagnosed by a physician based on auscultatory findings of crepitations or bronchial breathing or the IMCI signs of "severe pneumonia" mentioned above. Thus, the diagnosis "clinical pneumonia" is likely to be more specific and represent a more severe illness than the IMCI's operational definition of pneumonia (or ALRI) (130). Unfortunately, the various trials on pneumonia use these definitions somewhat inconsistently (13, 30, 73, 75, 124, 131).

**Treatment of lower respiratory infections with zinc.** There is limited information on zinc as adjunct therapy for ALRI and pneumonia. No effect of zinc administration on the enrollment episode was observed in the two clinical trials from which results are available (73, 132). It should be noted that these two trials together included only 236 subjects, and probably yielded an insufficient power to identify treatment effects of clinical and public health importance.

**Prevention of lower acute respiratory infections with zinc.** The above-mentioned analysis of preventive zinc supplementation trials also included data for ALRI from four trials (13). The pooled analysis of data from India (131), Vietnam (124), Peru (75) and Jamaica (30) showed that routine zinc supplementation reduced the odds of pneumonia with 41% (95% CI 17%, 69%).
Treatment and prevention of malaria

A recently completed multicenter trial in Ecuador, Ghana, Tanzania, Uganda, and Zambia assessed zinc as adjunct therapy during *Plasmodium falciparum* malaria in 1,087 children aged 6 to 60 months (133). This study concluded that: “Zinc does not appear to provide a beneficial effect in the treatment of acute, uncomplicated falciparum malaria in preschool children”. A trial in 109 Gambian children showed a non-significant reduction in clinic visits for *P. vivax* malaria in those that were given 70 mg elemental zinc twice weekly for 65 weeks compared to the placebo recipients (134). In a randomized placebo-controlled trial of 274 preschool children in Papua New Guinea, there was a 38% (95% CI 3%, 60%) reduction in the risk of febrile episodes associated with *P. falciparum* malaria in children receiving daily zinc supplementation (135). The effect of zinc was higher in those that had heavy parasitemia, indicating that poor zinc nutriture increases the severity. However, in a trial in almost 700 six to 31 months old children in Burkina Faso, those receiving zinc experienced fewer diarrheal episodes but not fewer episodes of malaria than those in the placebo group (74). Thus, the results from the trials that have assessed the effect of oral zinc against malaria have yielded conflicting results. Adequate zinc nutrition may not be as important in malaria as it seems to be in diarrhea and pneumonia.

Can improvement in zinc nutriture reduce the high mortality in children of developing countries? This seems likely based on the discussion above and it is supported by a trial in 1,154 small for gestational age (SGA) children in India (136). In this trial, placebo or 5 mg elemental zinc as zinc sulfate was given daily between 30 and 284 days of age. There were five fatalities among the zinc supplemented and 15
among the placebo recipients, the relative risk (RR) of dying between those in the zinc and those in the placebo group was accordingly 0.32 (95% confidence interval: 0.12-0.89). The results from this trial and the trial that demonstrated a reduced death risk in children belonging to clusters that received zinc as adjunct therapy for acute diarrhea (119) are, indeed, promising. Thus, zinc is a candidate for adjunct therapy in common childhood infections, for routine supplementation, and/or food fortification in order to prevent such infections and to promote growth. Before zinc reaches child health programs, however, sufficient evidence regarding the positive effects, as well as any adverse effects should be sought. Large trials to measure whether and to what extent routine zinc supplementation reduces childhood mortality are underway in India, Tanzania and Nepal. If routine zinc indeed results in reduced mortality in children of developing countries, there is a need to develop and implement strategies to improve zinc nutriture. These include dietary diversification / modification to enhance the availability, access and utilization of zinc dense foods, food fortification and supplementation. This thesis will not deal with the different strategies to promote an adequate intake of zinc and other micronutrients, but emphasis hereby that, although routine zinc supplementation may give substantial health benefits, aspects of affordability, access, equity and sustainability may favor food-based approaches to improve childhood nutrition including zinc nutrition worldwide.
Objectives

The overall objective of the thesis work was to assess the importance of zinc in common childhood infections. The specific objectives were to

1. in young children of a developing country;
   1.1. assess whether zinc administration during acute diarrhea reduces the severity of the episode,
   1.2. assess whether vitamin A administration is a prerequisite for the therapeutic effect of zinc during acute diarrhea,
   1.3. assess whether zinc supplementation during acute diarrhea is as effective when delivered by caretakers as when given by trained health workers,
   1.4. assess whether zinc supplementation during acute diarrhea prevents common infections for a month after cessation of the episode,
   1.5. assess whether infection severity is associated with plasma zinc concentration during the first days of acute diarrhea,
   1.6. assess whether routine zinc supplementation reduces the incidence and prevalence of respiratory infections,

2. in mice;
   2.1. examine whether zinc depletion is associated with reduced immune responses to pneumococcal antigens,
   2.2. examine whether zinc depleted individuals have an increased infection severity following mucosal pneumococcal challenge, and to
2.3. assess whether immunization before challenge with *Streptococcus pneumoniae* can reduce the infection severity and the inflammation-associated depletion of zinc from plasma and bone.
Methods

Two field trials and two animal experiments are included in this thesis work. In the two clinical trials conducted in Nepal and India, we included 1,792 and 2,482 children, respectively. Eighty mice were used in the two animal experiments.

Field trials

In Nepal, 6 to 35 months old children with acute diarrhea were randomly allocated to receive placebo, zinc, or zinc and vitamin A during the illness. The predefined outcomes were duration of the illness, stool frequency and the proportion of episodes lasting for more than 3, 7 or 14 days after enrollment. In India, seemingly healthy children aged 6 to 30 months, were randomized to receive placebo or zinc daily for four months. Outcomes were episodes of ALRI and clinical pneumonia. The same placebo syrup, zinc syrup and bottles were used in the two trials and the randomization lists were generated and kept at the Statens Serum Institute in Copenhagen, Denmark. The essential aspects of these two trials are listed in table 1 and papers I and III.
Table 1

### Clinical Trials - Human Experiments

<table>
<thead>
<tr>
<th>Objective</th>
<th>Nepal - Zinc as adjuvant therapy during acute diarrhea</th>
<th>India - zinc supplementation for prevention of ALRI and pneumonia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Assess the efficacy and effectiveness of oral therapeutic zinc on the outcome of acute diarrhea</td>
<td>Asses the efficacy of routine zinc administration for the prevention of ALRI and pneumonia</td>
</tr>
<tr>
<td>Design</td>
<td>Randomized placebo controlled trial with three double masked and one open group</td>
<td>Randomized placebo controlled double masked trial</td>
</tr>
<tr>
<td>Study site</td>
<td>Entire urban community (Bhaktapur)</td>
<td>Urban slum (New Delhi)</td>
</tr>
<tr>
<td>Age at enrollment</td>
<td>6-35 months</td>
<td>6-30 months</td>
</tr>
<tr>
<td>Study groups</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Group size</td>
<td>~450</td>
<td>1241</td>
</tr>
<tr>
<td>Total</td>
<td>1792</td>
<td>2482</td>
</tr>
<tr>
<td>Inclusion criteria</td>
<td>Plan to live in the study area</td>
<td>Plan to live in the study area</td>
</tr>
<tr>
<td></td>
<td>Passage of ≥3 loose or watery stools 24 hours prior to enrollment</td>
<td>Informed consent available from at least one of the caretakers</td>
</tr>
<tr>
<td></td>
<td>Preenrollment duration &lt;96 hours</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Informed consent available from at least one of the caretakers</td>
<td></td>
</tr>
<tr>
<td>Exclusion criteria</td>
<td>Received massive dose of vitamin A in the past 4 weeks</td>
<td>Received massive dose of vitamin A in the past 2 months</td>
</tr>
<tr>
<td></td>
<td>Disease requiring hospitalization</td>
<td>Disease requiring hospitalization</td>
</tr>
<tr>
<td></td>
<td>Included previously &lt; 4 months ago</td>
<td>Included previously</td>
</tr>
<tr>
<td>Randomization</td>
<td>individual</td>
<td>individual</td>
</tr>
<tr>
<td>Block size</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>Intervention</td>
<td>Vitamin A bolus at enrollment</td>
<td>Vitamin A bolus at enrollment</td>
</tr>
<tr>
<td></td>
<td>Infants: 100,000 IU</td>
<td>Infants: 100,000 IU</td>
</tr>
<tr>
<td></td>
<td>Toddlers: 200,000 IU</td>
<td>Toddlers: 200,000 IU</td>
</tr>
<tr>
<td></td>
<td>Daily zinc until 1 week after recovery</td>
<td>Daily zinc for 4 months</td>
</tr>
<tr>
<td></td>
<td>Infants: 15 mg</td>
<td>Infants: 10 mg</td>
</tr>
<tr>
<td></td>
<td>Toddlers: 30 mg</td>
<td>Toddlers: 20 mg</td>
</tr>
<tr>
<td>Co-interventions</td>
<td>Antibiotics if required (e.g. dysentery)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Infants: 100,000 IU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toddlers: 200,000 IU</td>
</tr>
<tr>
<td>Follow-up</td>
<td>Every 5th day until recovery</td>
<td>Every 7th day for four months</td>
</tr>
<tr>
<td></td>
<td>Registration of clinic visits</td>
<td>Registration of clinic visits</td>
</tr>
<tr>
<td></td>
<td>One month after recovery</td>
<td></td>
</tr>
<tr>
<td>Outcomes</td>
<td>Duration of diarrhea</td>
<td>Episodes of ALRI and pneumonia</td>
</tr>
<tr>
<td></td>
<td>Cases lasting &gt;3, &gt;7 and &gt;14 days</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stool frequency</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Morbidity for one month after recovery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Regurgitation and vomiting</td>
<td></td>
</tr>
<tr>
<td>Described in</td>
<td>Paper I &amp; Paper II</td>
<td>Paper III</td>
</tr>
</tbody>
</table>
**Ethical issues.** The All India Institute of Medical Sciences ethics committee approved the study in India. The study in Nepal had ethical clearance from the Nepal Health Research Council in Kathmandu, and the Human Research Ethical Committee of the Medical Faculty at the University of Bergen. The implementation of all aspects of the studies was in agreement with the international ethical guidelines for research involving human subjects as stated in the latest version of the Helsinki Declaration. Informed and, when possible written, consent was obtained from at least one of the parents.

**Zinc syrup.** The placebo syrup [1 kg syrup contained 597 g water, 400 g sugar, 1.2 g peach flavor no. 061508* (Einar Willumsen, 23-25 Abildager, Brøndby, Denmark), 1 g methylparabene, 1 g xanthan gum, 0.15 g saccharin sodium] and the zinc syrup were identical in appearance. We used zinc gluconate as the zinc salt. The taste and acceptability of the syrup was assessed in Scandinavian and Indian adults and children prior to manufacture. The highest concentration at which the taste of the placebo and zinc syrup was identical was 2.5 mg elemental zinc per ml, although even further dilution could not mask a slight metallic aftertaste. We accordingly used this concentration in the zinc syrup. In Nepal, 6 and 12 mL (15 and 30 mg zinc) and in India, 4 and 8 mL (10 and 20 mg zinc) were given to infant and toddlers, respectively.

**Geographical distribution of the enrolled cases.** The geographical distribution of the children’s homes in Nepal was visualized using a global positioning system (GPS)-based computerized plot (Map). The homes of the children that resided outside the city are not included. The map shows that the residencies of the enrolled children were evenly distributed throughout the target area.
Predictors of plasma zinc

In the cross-sectional study described in paper II, we used the baseline data from the field trial in Nepal described in paper I. We selected several relevant variables of illness history, socioeconomic status, clinical findings and biochemical variables and assessed their associations with the plasma zinc concentration in crude and multiple linear regression analysis.
Mouse models

The animal experiments addressed objectives 2.1, 2.2 and 2.3, i.e. whether zinc depleted mice have reduced immune responses to pneumococcal antigens, whether zinc depleted mice have an increased infection severity following mucosal pneumococcal challenge, and whether immunization of mice with pneumococcal surface protein A (PspA) before challenge with *S. pneumoniae* can reduce the infection severity and the inflammation-associated depletion of plasma and bone zinc. Details of the two experiments are shown in table 2.

Table 2

<table>
<thead>
<tr>
<th>Mouse experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
</tr>
<tr>
<td><strong>Objective</strong></td>
</tr>
<tr>
<td><strong>Design</strong></td>
</tr>
<tr>
<td><strong>Groups</strong></td>
</tr>
<tr>
<td><strong>Group size</strong></td>
</tr>
<tr>
<td><strong>Number of mice</strong></td>
</tr>
<tr>
<td><strong>Diet zinc levels</strong></td>
</tr>
<tr>
<td><strong>Feeding time</strong></td>
</tr>
<tr>
<td><strong>Intervention to all mice</strong></td>
</tr>
<tr>
<td><strong>Outcomes</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Described in</strong></td>
</tr>
</tbody>
</table>
The study protocols of the animal experiments were approved by the local officer of the Experimental Animal board under the Norwegian ministry of Agriculture, and the experiments were in conformity with the laws and regulations controlling experiments with live animals in Norway.

The pair feeding. Zinc depletion affects growth and results in reduced appetite and taste, which may cause reduction in the intake of other nutrients as well. The mice in the zinc depletion experiments were therefore pair-fed to ensure similar intake of all nutrients except of zinc. The consumed fodder of the zinc depleted mice was accordingly estimated and the intake of the other mice was restricted to the body-weight-adjusted intake of the zinc depleted mice. All mice were therefore weighed and the fodder consumed was estimated for 24- or 48-hour periods throughout the studies. We used special cages where the food intake could be assessed and controlled. The cages had stainless steel meshed floor to prevent recycling of zinc from body wastes, and feeding chambers to determine the food consumption. To reduce exposure to environmental zinc, we washed the cages with 10% HCl before use and with deionised water at regular intervals throughout the study. Pair-fed mice were housed individually.

Statistical methods

Statistical analyses were undertaken using the statistical and data management package Stata®, version 6 and 7 (StataCorp, College Station, TX, USA) and SAS version 8.1 (SAS Institute, Cary, NC, USA). In the trial in Nepal (Paper I and II), we calculated Z scores weight-for-age, length-for-age, and weight-for-length using LMS values (137-
obtained from the Centers for Disease Control and Prevention growth charts (141). In the trial in India (Paper III), we calculated these values using EPI INFO version 6. All analyses of the outcomes from the clinical trials were carried out on an intent-to-treat basis.

PAPER I

We used regression analysis with correction for repeated entries of the same child using generalized estimation equations (GEE) with an exchangeable variance-covariance structure, i.e. a random effects model (142). Relative risks (RR) were calculated using generalized linear models with the binomial distribution family and a logarithmic link function. For calculating the absolute risk reduction of prolonged or persistent diarrhea we used the binomial distribution and an identity link function. The estimates in these analyses were also adjusted for breastfeeding status and age categories in addition to the adjustment for repeated entries. Log-transformed total number of stools during the first 4 days were compared between the groups using generalized linear models with GEE, values were antilogged, and the results were expressed as geometric mean or the ratios of geometric means compared with the placebo group. In the Cox proportional hazards model, we used the COVSANDWICH option in SAS to obtain a robust estimate of the variance, in order to account for repeated observations in the same child. Adjustment for ties was done by the DISCRETE option in SAS.

PAPER II

In this paper, we used Stata® version 7 for the analyses. The plasma zinc levels were symmetrically distributed and the association between plasma zinc and the variables of interest were assessed in simple and multiple linear regression analyses. We assessed
the associations between several variables and plasma zinc. Based on these analyses, we selected variables for the multiple regression models as described elsewhere (143). Differences between the groups were expressed as the mean differences with the corresponding 95% CIs. All independent variables except albumin and axillary temperature were categorical; indicator variables were used to assess the effect of variables with more than two categories. We also assessed a number of relevant interactions. One hundred and ninety-nine children were included in the study more than once; adjustment for repeated entry of the same child was undertaken using GEE with an exchangeable variance-covariance structure. The correlation between C-reactive protein (CRP) and measured temperature was undertaken using Spearman rank-order correlation. The graph describing the relation between plasma zinc and axillary temperature in the three CRP categories was constructed using locally weighted scatterplot smoothing by the Stata® ksm command with the option “lowess” and a bandwidth of 0.8.

PAPER III

We compared the risk of and incidence of ALRI and clinical pneumonia in the two groups and calculated absolute risk reductions with their 95% CIs. For the person time analyses, the denominator was the days of which reliable information about illness was available. To account for the correlation of multiple episodes in the same child, we used GEE with an exchangeable variance-covariance structure. The surveillance data for each child were divided into eight child periods of 15 days each. In the GEE model, occurrence of a new episode of ALRI and clinical pneumonia in a child period were modeled as binomial dependent variables from which we calculated the ORs between the zinc group and placebo group. The analyses were undertaken with Stata® version 6.
PAPER IV
In this paper we used the Kruskal-Wallis equality of populations rank test for estimating the statistical significance of the differences in the fold-rise of specific IgM concentrations between the experimental groups. For comparing the mean zinc concentrations and the mean body weights between the experimental groups, we used one-way ANOVA with the Bonferroni post hoc test. Correlation was assessed using Spearman rank-order correlation. The analysis was undertaken with Stata® version 7.

PAPER V
Means, standard deviations, differences of means, and 95% CI and \( t \)- tests were used to describe and compare continuous variables. Immune responses were log-transformed to achieve symmetrical distributions, and \( t \) tests assuming unequal variances of the log-transformed values were used in the analyses. Bacterial counts and their log-transformations were not normally distributed, and medians and interquartile ranges were calculated, while the Wilcoxon rank-sum (Mann-Whitney U) test was used for comparisons. Proportions were compared with Fisher’s exact test, and RRs were calculated to describe the differences between the experimental groups. If no event was observed in a cell, the value of that cell was set to 1 to calculate an underestimated RR.
Synopsis of the papers

PAPER I - Effectiveness and efficacy of zinc for the treatment of acute diarrhea in young children

**Background:** More information on the efficacy, effectiveness and possible adverse effects of zinc used as adjunct therapy for acute childhood diarrhea is required before recommending large-scale programmatic implementation. Furthermore, a clarification of whether beneficial effects of zinc depend on concomitant vitamin A administration is needed.

**Objectives:** To measure the effectiveness and efficacy and identify any side effects of giving 3 RDA (125) elemental zinc orally to 6-35 months old children with acute diarrhea and to assess whether the effect of zinc is dependent on concomitant vitamin A administration. Moreover, we wanted to assess whether zinc given during diarrhea had an effect on morbidity for one month after termination of the enrollment episode.

**Methods:** Double blind randomized-placebo-controlled trial enrolling 1,792 cases of acute diarrhea in Nepalese children. There were four study groups; three groups were masked and the children supplemented daily by field workers with placebo syrup, zinc syrup or zinc syrup and a massive dose of vitamin A at enrollment. The fourth group was open and the caretaker gave the children zinc syrup daily. The syrup was given for 7 days after recovery. Day-wise information on morbidity was obtained on household visits every fifth day until recovery (*table 3*).
Table 3

**Intervention groups in a randomized trial on the efficacy and effectiveness of therapeutic zinc on the outcome of acute diarrhea in Nepalese children 6-35 months of age.**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Placebo</th>
<th>Zinc</th>
<th>Vitamin A-zinc</th>
<th>Caretaker-zinc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time of delivery:</strong></td>
<td>Masked by trained field workers:</td>
<td>Masked by caretaker:</td>
<td>Masked</td>
<td>Open</td>
</tr>
<tr>
<td>Enrolment day</td>
<td>Placebo capsule</td>
<td>Placebo capsule</td>
<td>Vitamin A* capsule</td>
<td>Placebo capsule</td>
</tr>
<tr>
<td>Daily to 7 days after recovery</td>
<td>Placebo</td>
<td>Zinc*</td>
<td>Zinc*</td>
<td>Zinc*</td>
</tr>
</tbody>
</table>

*Children less than 12 months of age received 100,000 IU vitamin A at enrolment and 15 mg elemental zinc while toddlers received 200,000 IU vitamin A at enrolment and 30 mg elemental zinc daily.

**Results:** As compared to the placebo group, the relative hazards for termination of diarrhea were 26% (95% CI 8%, 46%), 21% (4%, 38%) and 19% (2%, 40%) higher in the zinc, zinc-vitamin A and zinc-caretaker groups, respectively. The relative risks for having an episode that lasted >7 days after enrollment in these groups were 0.57 (0.38, 0.86), 0.53 (0.35, 0.81) and 0.55 (0.37, 0.84).

After this paper was published, we reanalyzed the data measuring the effect of zinc administration on prolonged diarrhea (now defined as total duration of an episode ≥7 days) or persistent diarrhea (total duration of an episode ≥14 days) In this analysis, diarrhea duration included days with diarrhea prior to enrollment rather than only duration after enrollment. The results of this analysis are shown in table 4 where the risk of prolonged and persistent diarrhea is compared between the zinc-efficacy group or a group consisting of all children that were given zinc by the field workers on the one hand and the placebo group on the other.
Table 4

Effect of daily zinc administration on prolonged diarrhea (episode lasting for ≥7 days) or on persistent diarrhea (episode lasting for ≥14 days) in children 6 - 35 months of age

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=449)</th>
<th>Zinc (n=442)</th>
<th>Pooled Zinc (n=889)**</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prolonged diarrhea</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (%)</td>
<td>135 (30.4)</td>
<td>135 (30.4)</td>
<td>135 (30.4)</td>
</tr>
<tr>
<td>RR†</td>
<td>0.75 (0.60, 0.93)</td>
<td>0.78 (0.65, 0.93)</td>
<td></td>
</tr>
<tr>
<td>ARR*</td>
<td>0.08 (0.02, 0.13)</td>
<td>0.07 (0.02, 0.12)</td>
<td></td>
</tr>
<tr>
<td>NNT¶</td>
<td>13 (8, 50)</td>
<td>15 (9, 66)</td>
<td></td>
</tr>
<tr>
<td><strong>Persistent diarrhea</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cases (%)</td>
<td>23 (5.2)</td>
<td>23 (5.2)</td>
<td>23 (5.2)</td>
</tr>
<tr>
<td>RR†</td>
<td>0.57 (0.29, 1.1)</td>
<td>0.60 (0.35, 1.0)</td>
<td></td>
</tr>
<tr>
<td>ARR*</td>
<td>0.025 (0.0003, 0.05)</td>
<td>0.025 (0.002, 0.045)</td>
<td></td>
</tr>
<tr>
<td>NNT¶</td>
<td>40 (20, 3333)</td>
<td>40 (22, 500)</td>
<td></td>
</tr>
</tbody>
</table>

†RR=relative Risk, *ARR = Absolute risk reduction, ¶NNT= Numbers needed to treat, i.e. to prevent 1 case of prolonged and 1 case of persistent diarrhea we need to treat 13 and 40 children, respectively. **The pooled zinc group consists of children in the zinc efficacy and zinc vitamin A groups.

Five percent and 5.1% of all syrup administrations were followed by regurgitation (defined as regurgitation of the supplement within 15 minutes after ingestion) in the zinc and in the zinc-vitamin A group, respectively, while this occurred after only 1.3% of the placebo administrations. Vomiting during diarrhea was also more common in children receiving zinc. Eight hundred and thirty-two of the enrolled children made 1,209 visits to our field clinic during one month after termination of the enrollment episode, the number of visits and the number of children who visited the clinic were similar in the children belonging to the 4 intervention groups. Furthermore, there were no differences in caretaker-reported morbidity, the number of physician visits or hospitalizations during a one-month period after recovery from the diarrheal episode.
**Interpretation:** Three RDA of zinc given daily by caretakers or by field workers substantially reduced the duration of diarrhea and caused some regurgitation and vomiting. The effect of zinc was not dependent on or enhanced by concomitant vitamin A administration. There was no measurable effect of zinc administration on subsequent morbidity for one month after recovery from the diarrheal episode.
PAPER II - Determinants of plasma zinc concentration in children with acute diarrhea

**Background:** Plasma and serum zinc concentration are the most widely used markers of zinc status in individuals and in populations.

**Objective:** To identify determinants of plasma zinc concentration during acute childhood diarrhea.

**Methods:** Cross-sectional study of 1,757 cases of acute diarrhea in 6 to 35 months old Nepalese children. The association between plasma zinc concentration and several clinical, anthropometric, socioeconomic and biochemical variables were estimated using simple and multiple linear regression analyses.

**Results:** An increase in the axillary temperature of 1 °C was associated with a reduction in the mean plasma zinc concentration of 0.59 (95% CI 0.44, 0.74) µmol/L. Dysentery and elevated plasma CRP concentration were also independently associated with lower plasma zinc levels. **Figure 1** shows the association of axillary temperature with the plasma zinc concentration within each of three CRP concentration categories. Children with dehydration had higher plasma zinc levels than those who were not dehydrated. Furthermore, a reduction in the plasma albumin concentration of 1 g/L was associated with a 0.25 (CI 0.21, 0.29) µmol/L decrease in zinc concentration. The plasma albumin levels confounded the associations between some clinical variables and plasma zinc concentration, but did not substantially influence the associations between
axillary temperature or dehydration and plasma zinc concentration. The measured plasma zinc concentration increased with increasing degree of observed hemolysis.

**Interpretation:** Dehydration and clinical and biochemical indicators of inflammation as well as hemolysis and, when possible, plasma albumin concentration should be taken into account when plasma zinc concentration is used as a proxy for zinc status during childhood diarrhea.

**Figure 1**

Plasma zinc concentration and axillary temperature in Nepalese children with acute diarrhea by three categories of plasma CRP (<8, 8-15 and >15 mg/L). The graph was made using locally weighted scatterplot smoothing using the ksm command with the “lowess” option in Stata® 7.
PAPER III - Effect of routine zinc supplementation on pneumonia in children aged 6 months to 3 years: randomized controlled trial in an urban slum

Background: The effect of routine zinc supplementation on the incidence of ALRI and clinical pneumonia needs to be determined.

Objectives: To evaluate the effect of daily zinc supplementation on the incidence of ALRI and clinical pneumonia.

Methods: Double blind, randomized placebo controlled trial in a slum community in New Delhi, India. Children aged 6 to 30 months (n=2,482) were randomized to receive placebo or zinc every day for four months. Infants received 10 mg and toddlers 20 mg elemental zinc. All children received a single massive dose of vitamin A (100 000 IU for infants and 200 000 IU for toddlers) at enrolment. The households were visited weekly. Any child with cough and LCI or respiratory rate > 5 breaths per minute less than the WHO defined cut-off value for fast breathing were brought to the study physicians for a detailed clinical investigation.

Results: The proportion of children who had ALRI or the incidence of ALRI during follow-up was not different in the two groups. Zinc supplementation resulted in a reduced risk of clinical pneumonia (absolute risk reduction 2.5%, 95% CI 0.4%, 4.6%). After correction for multiple episodes in the same child by GEE, the OR for clinical
pneumonia was 0.74 (95% CI 0.56, 0.99). There was a significant increase in the average number of days with vomiting in the zinc group compared to the placebo group (4.3 (SD 5.8) v 2.6 (SD 3.9); difference in means 1.7, 95% CI 1.3, 2.1).

**Interpretation:** Four months daily zinc supplementation substantially reduced the incidence of clinical pneumonia in children who had received a massive dose of vitamin A.
PAPER IV - The immune response to pneumococcal polysaccharide vaccine in zinc depleted mice

**Background:** Several experiments have described dramatic effects of zinc depletion on almost all facets of the immune system.

**Objective:** In mice, assess the effect of zinc deprivation on the immune response to the pneumococcal polysaccharide antigens in the commercially available Pneumovax® pneumococcal vaccine.

**Methods:** Young female BALB/c mice were fed diets with 2.7, 5.8 or 25 µg elemental zinc per mg diet for seven weeks.

**Results:** There were significant differences in the mean body weights between the feeding groups and we demonstrated a dose response of the zinc level in the diet on weight gain. The induced zinc deficiency had no measurable effect on the anti-pneumococcal polysaccharide IgM response following immunization with the pneumococcal vaccine.

**Interpretation:** Although zinc depletion has detrimental effects on the immune system, we could not identify a deprivation of the T cell independent response to pneumococcal polysaccharide capsule antigens.
PAPER V- Effects of zinc deficiency and pneumococcal surface protein A immunization on zinc status and the risk of severe infection in mice

**Background:** Zinc deficiency is associated with an increased risk of acute respiratory tract infections, which can be reduced by daily zinc administration. PspA is present on all virulent strains of *S. pneumoniae* (144). Immunization with this protein protects animals against sepsis, intratracheal infections, and, when administrated mucosally, carriage of *S. pneumoniae* (145-147). PspA is considered to be a promising vaccine candidate for prevention of pneumococcal disease in humans (148).

**Figure 2**

**Objective:** To assess the effect of zinc depletion on the severity of pneumococcal infection and on the immune response to PspA and to assess whether PspA immunization reduces infection severity and inflammation-associated zinc redistribution.
**Methods:** Young female BALB/c mice were pair fed with 2.0 or 25 µg elemental zinc per mg diet for four weeks (Table 2, **Figures 2 and 3**). We immunized two groups of mice with PspA parenterally twice 14 days apart and challenged the mice intra-nasally with *S. pneumoniae* 14 days after the last immunization. We assessed the effect of the PspA vaccination and the zinc depletion on the severity of pneumococcal infection and on serum and bone zinc concentrations.

**Figure 3**

![Diagram of mouse experiment](image)

*Design of the mouse experiment described in paper V. One animal died following nozzle blockage before immunization started.*

**Results:** Mice on the zinc deficient diet showed substantially reduced immune responses to PspA, more extensive pneumococcal colonization in the nasal mucosa, more severe infections and an increased risk of death. PspA immunization reduced the risk of severe disease, the reduction in severity was reflected in substantially reduced zinc depletion from bones but not in a measurable reduction in the risk of death.

Among the zinc replete mice, PspA immunization reduced the depletion of zinc from plasma.
**Interpretation:** In mice, zinc depletion leads to increased severity of pneumococcal infection and risk of death following pneumococcal challenge. PspA immunization reduced the magnitude of the zinc redistribution.
Discussion

Treatment of acute diarrhea

Our findings confirm that oral zinc administration is highly efficacious in treating 6 to 35 month old children with acute diarrhea. The reduction in duration and the risk of prolonged illness was larger than reported in most previous studies (12, 69, 70, 79, 84, 120). Furthermore, a significant and substantial reduction in the risk of persistent diarrhea following zinc therapy during acute diarrhea has not previously been demonstrated. As nearly half of the 2-3 million diarrhea related deaths are in persistent diarrhea (113, 114), a large decrease in the risk of long lasting illness is of great public health importance. Indeed, despite the potential bias caused by the association between zinc administration and increased usage of ORS, a trial from Bangladesh showed a 51% reduction in non-injury fatalities in clusters where zinc was given to children with diarrhea (119, 123).

Our study demonstrates that the programmatically feasible approach of having caretakers giving this zinc syrup to the children is as effective as when given by trained field workers. However, although we had instructed the field workers to refrain from message delivery, the compliance in the caretaker group could still have been reinforced by the repeated morbidity visits. The effect of such reinforcement was probably limited and we observed that the effectiveness of zinc was evident already on the third day after enrollment, i.e. even before the first morbidity visit. It should be noted, however, that a design with group rather than individual randomization, such as
in the trial described in paper I may better measure program relevant effectiveness (149).

One 10 mg zinc tablet made by Nutriset (Malaunay, France) costs approximately 0.7 Nepalese rupees (NRS), if we assume a mean treatment time of seven days and a dose of 20 mg zinc, the price of a course during acute diarrhea will be NRS 10 (€ 0.11). According to the results from the study in paper I, the numbers needed to treat (NNT) to prevent one case of prolonged (total episode duration ≥ 7 days) or persistent (total episode duration ≥ 14 days) diarrhea are 13 and 40 children, respectively. The corresponding drug costs are accordingly NRS 130 (€ 1.4) and NRS 400 (€ 4.4) for preventing one episode of prolonged or persistent diarrhea. These estimates are probably too high as locally prepared zinc remedies are likely to be cheaper. The costs incurred by the health system beyond that of the remedy is likely to marginal as our study shows that mothers are excellent providers of zinc therapy. Thus, oral zinc seems to be a feasible candidate to reduce diarrheal morbidity and possibly mortality in developing countries. Antibiotics, which are indicated in some diarrheal syndromes such as cholera and bacterial or amoebic dysentery, are overused and may, in fact, be harmful to the individual child. Because diarrhea is so common, such misuse contributes substantially to the spread of antibiotic resistance. Cheap oral zinc has the potential to replace misused antibiotics during acute diarrhea and may thus contribute to preventing antibiotic resistance.

The effect of zinc was not dependent on concomitant vitamin A administration. A possible interaction between vitamin A and zinc, however, may have been masked by the high pulse vitamin A coverage in Nepal. Bhaktapur is a farming community, it is
located in one of the more fertile areas of Nepal and a wide variety of foods including green leafy vegetables are available. Indeed, in the past years, there have been very few reports of cases of vitamin A deficiency from the Kathmandu valley (personal communication, R. K. Adhikari). We can accordingly not rule out the possibility that in populations where vitamin A deficiency is common, the therapeutic effect of zinc during diarrhea is dependent on or enhanced by concomitant vitamin A administration. However, any pharmacological effect of a high dose of vitamin A given with zinc early in the disease would not necessarily be affected by adequate vitamin A status and was not observed in our study.

**Delayed effects of zinc administration**

In the study described in paper I, we recorded morbidity for one month after recovery from the diarrheal episode and were not able to detect any differences between the study groups. This is in contrast to other trials (13, 80, 100) that have shown an impact of short-term zinc administration on subsequent diarrheal morbidity of almost the same magnitude as that of the studies measuring morbidity during routine zinc supplementation. Furthermore, it is likely that any delayed effect should have been strongest during the time immediately after zinc supplementation and this effect should therefore have been detectable during our one month observation. Our finding of no delayed effect of therapeutic oral zinc is supported by another similar trial on zinc during acute diarrhea in 1,219 Indian children (120). This trial showed that therapeutic zinc administration substantially reduced the duration of the diarrheal episodes but there was no effect on the subsequent one-month morbidity (M. K. Bhan, personal communication). In the trial in India (120) and the trial described in paper I, the one
month morbidity assessment was based on one interview. We asked about days with diarrhea, cough, difficult breathing and fever and whether the children had visited a physician or been hospitalized during the month after cessation of the diarrheal episode. Although these outcomes are not highly specific for diarrhea and ALRI, and the fact that low specificity tends to shift the effect measures towards the null (149), these two large studies raise some doubts about the long-term beneficial effects of short-term zinc supplementation.

**Prevention of acute lower respiratory infections**

Zinc supplementation did not have a substantial impact on the prevalence of ALRI. The odds of clinical pneumonia, however, was reduced by 26% through daily zinc supplementation for 4 months. This is in line with the results from other trials that have shown that routine zinc administration may prevent severe rather than mild illnesses (13, 85, 131, 150). Bacteria such as *S. pneumoniae* and *H. influenzae* cause a substantial proportion of clinical pneumonias (151, 152). The finding that daily zinc administration reduces the risk of severe ALRI, such as clinical pneumonia, match the finding from the study described in paper V that, in mice, zinc deficiency was associated with more severe infections and increased lethality following challenge with *S. pneumonia*. The lack of effect of routine zinc administration on mild ALRI, however, could also in part be explained by the relatively low specificity of the criteria for ALRI.
Masking of the clinical trials

Blinding posed some challenge, particularly because of the metallic aftertaste and increased frequency of regurgitation and vomiting. The syrup bottles and capsules were packed separately for each study participant and the random allocation of the serial numbers were done before the studies were initiated. Although we have no information to that effect, the staff may have attempted to guess the group identity of the children that regurgitated, which again could have influenced the way morbidity was recorded. We believe, however, that the masking worked well and any differential misclassification of outcomes kept to a minimum by detailed protocols and rigid training and re-training sessions before and during the studies. In the acute diarrhea study in Nepal, the field workers and the families knew that the children of the effectiveness group were given zinc and not placebo (Paper I). Children in the effectiveness group did apparently not have a different effect of zinc compared with those in the two efficacy groups. Thus, the effect did not seem to have been altered because of the knowledge of group identity. Furthermore, exclusion of those who experienced regurgitation did not reduce the estimated effect of zinc administration during acute diarrhea (data not shown).

Plasma zinc, plasma albumin, infection and inflammation

The inverse correlation between plasma zinc concentration and the severity of inflammation confirms earlier studies in humans and in animals (54-56, 59, 60, 153). This is to our knowledge the first study to demonstrate this association in a sample of children suffering from common infections such as acute diarrhea. Furthermore, the correlation between body temperature and plasma zinc concentration has not previously
been described and supports the hypothesis stated by Brown that “fever might be a useful marker of whether or not a particular infection is affecting the plasma zinc concentration” (59). We believe that the associations between plasma zinc and markers of inflammation were due to inflammation-associated zinc redistribution. This is supported by the findings in paper V, which shows that in mice, PspA immunization was associated with reduced infection severity, higher bone zinc concentrations and among the zinc replete mice, higher serum zinc levels. Cousins and coworkers showed that interleukin-1, which is secreted by phagocytic cells during infections, increased the zinc uptake in the liver, bone marrow, and in the thymus (61). In the gut, skin and plasma, however, the zinc concentration decreased. It is possible that this redistribution of zinc decreases the resistance to infections. The role of zinc on the resistance to infections is not only mediated through the acquired immune response (14, 34). For example, zinc has probably several roles in the normal function of the respiratory (50) and gastrointestinal tract (48, 49) and it is important for phagocytosis (35-38). Thus, severe infections may cause a local impairment in the resistance to infections by zinc redistribution, creating a vicious cycle of zinc redistribution and severe infection. If this theory is correct, it may, in part, explain why routine zinc supplementation is more efficacious in preventing severe illnesses (paper III), i.e. illnesses followed by severe zinc redistribution, rather than mild illnesses. A clinical trial that included fever and LCI in the definition of ALRI demonstrated a beneficial effect of routine zinc administration on ALRI incidence (131). There was only a small non-significant effect on ALRI incidence when fever and LCI were left out from this definition. If fever is an indication of whether an infection causes inflammation-associated zinc redistribution, and if the impact of zinc is higher when such redistribution is extensive, it can explain why zinc was found to be efficacious only in preventing ALRI with fever. However,
this difference in the effect of zinc can also be explained by the consequence of using outcomes with low specificity, as discussed above. If severity is an effect-modifier for the therapeutic effect of zinc due to zinc redistribution, and if the plasma zinc depletion is paralleled in other tissues vital for host defense, plasma zinc levels could predict the response to zinc therapy. In the previously mentioned pooled analysis of the effect of zinc during acute and persistent diarrhea, there was a tendency, although statistically not significant, of a greater effect of zinc therapy in the subgroups with a zinc concentration below the median value (12).

Entry into the proposed vicious cycle of zinc redistribution and infection severity is more likely in those with impaired zinc nutriture. Individuals with suboptimal zinc status may thus face severe consequences of infections associated with severe inflammation such as those caused by *S. pneumonia*. Indeed, a high burden of pneumococcal infections is encountered in populations where zinc deficiency is prevalent. Children, especially in South Asia and other developing regions (10), the elderly (32, 46, 154), and those with sickle cell disease (SCD) (155) are at risk of zinc deficiency as well as of severe pneumococcal disease (128). The hemolysis in SCD causes hyperzincuria and subsequently zinc deficiency (155). Furthermore, SCD may cause autospleenectomy resulting in further increased susceptibility to invasive pneumococcal infections. Zinc administration in children with SCD have resulted in lower incidence of bacterial infections and decreased number of hospitalizations (156).

It is estimated that more than half of the pediatric clinical pneumonia cases in developing countries are caused by *S. pneumonia* (151, 152), which probably accounts for over 1 million deaths each year in children under 5 years of age (126, 128). In mice,
zinc depletion led to an increased risk of severe infection and increased lethality following challenge with *S. pneumoniae* (Paper V), a finding which supports a previous study (157). If this increased pneumococcal infection severity in zinc deficiency is mirrored in children, it is plausible that oral zinc administration may be used as adjunct therapy in pediatric pneumonia in developing countries. The hypothesis of a possible vicious cycle of zinc redistribution and infection severity renders a therapeutic effect of zinc in pneumonia even more probable. The lack of effect seen in the trials where zinc was given to children with ALRI (73, 132) may be due to inadequate sample sizes, too mild illnesses among the study participants, or lack of specificity of the outcomes. More studies on this important issue is required.

Plasma albumin levels were highly correlated with plasma zinc levels in children with acute diarrhea. Conditions associated with altered plasma volume also change the plasma albumin concentration and albumin levels may be lower during inflammation (158). Furthermore, malnutrition and zinc deficiency may also lead to albumin loss and reduced albumin syntheses (159, 160). In the plasma, zinc is bound to proteins, particularly albumin (10). It is therefore not surprising that the plasma zinc and albumin levels are highly correlated. The associations we described between plasma zinc and infection and albumin have implications for the interpretation of plasma or serum zinc levels in samples that have been collected from children with infections such as diarrhea.

Caution is required when attempting to draw conclusions regarding the direction of the causal pathway from cross-sectional studies such as the one described in paper II. Inflammation may reduce plasma zinc levels or children with low plasma zinc levels
may have more severe infections accompanied with higher temperature and elevated CRP. Zinc deficiency causes stunting and underweight (16). Being stunted, wasted or underweight, however, were not associated with having dysentery, fever or elevated CRP in the study described in paper II. Malnutrition with its frequently accompanied zinc deficiency (16) did accordingly not seem to lead to conditions that caused elevated CRP or increased temperature. This indicates that an increased inflammation resulted in reduced plasma zinc levels rather than the reverse. Studies in humans and animals (55, 57-59) also support our assumption that inflammation reduces plasma zinc levels.

Furthermore, the reduction in serum zinc levels have been found to be proportional to the level of induced parasitemia and with the dose of administered bacterial endotoxin (57, 58). The plasma zinc levels in our study were low (Paper I and II). We do not know whether the associations between markers of inflammation and plasma zinc levels and between plasma albumin levels and zinc levels will be the same for populations with higher plasma zinc levels. Moreover, measurement of body temperature is most accurate when done rectally or orally. We assessed the axillary temperature, but the staff went through rigorous training before and during the trial. There is therefore no reason to believe that errors in measuring the temperature might have exaggerated the associations described in paper II.

We compared age, anthropometric indices, stool frequency 24 hours prior to enrollment and axillary temperature between children (1587) in whom we had estimated plasma CRP, albumin and zinc concentrations and those (205) that had one of these values missing. The differences between the groups of the selected variables were small (Table 5) and unlikely to grossly influence the associations described in paper II.
Table 5

Selected characteristics of subjects with known albumin, CRP and zinc concentrations and in those that at least one of these measurements are missing.

<table>
<thead>
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<th>No missing value</th>
<th>Any missing value</th>
<th>Percent difference</th>
</tr>
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<td></td>
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<td>205</td>
<td></td>
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<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>mean</td>
</tr>
<tr>
<td>Z score weight for length</td>
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<td>1.1</td>
<td>-1.4</td>
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<td>1.3</td>
<td>-1.1</td>
</tr>
<tr>
<td>Hemoglobin level (g/L)</td>
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<td>1.4</td>
<td>11.1</td>
</tr>
<tr>
<td>Age (months)</td>
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<td>15.5</td>
</tr>
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<td>Axillary temperature (°C)</td>
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<td>36.7</td>
</tr>
<tr>
<td>Stool frequency</td>
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<td>4.1</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Dose and vomiting

We used relatively large doses of zinc in both of the field trials described in this thesis. While children participating in most therapeutic trials of zinc during acute diarrhea received doses of around 2 RDA (12, 69, 71, 77, 84), we used 3 RDA (Paper I). The WHO recommends that children aged 6 to 35 months consuming a diet with low zinc bioavailability have a daily intake of 11 mg zinc (161). The recommendations for those consuming diets with higher bioavailability are less. Thus, if routine zinc supplementation to reduce disease burden and/or promote growth is to be recommended, it is likely that any health authority will advice 1 RDA or the above
mentioned WHO recommendations and not 2 RDA as we used in the study reported
upon in paper III.

The two field trials demonstrated that children receiving zinc experienced more
regurgitation (paper I) and vomiting (paper I and III) than children in the placebo
groups. This is supported by a study that gave 5 mg elemental zinc daily as zinc
sulphate or placebo to 61 six to 24 month old Jamaican children (30). In this trial there
was a statistically significant “longer duration of vomiting” among the zinc recipients.
The authors speculated, however, that this could have been a chance finding. Except for
this trial, zinc-induced regurgitation and vomiting has to our knowledge previously not
been reported in clinical trials in children. In the diarrhea study in Nepal, one third of
the regurgitations after syrup ingestion occurred on the first day. In addition to the fact
that the children may have been somewhat stressed in the field clinic, a more careful
dispensing by the field workers or caretakers or gastrointestinal adaptation to zinc may
explain the lower regurgitation frequency on the subsequent days. Fortunately, the
vomiting and regurgitation was not associated with serious health consequences or
dropouts.

Vomiting was recalled and recorded on the morbidity visits and in the diarrhea study
(Paper I), regurgitation was observed directly during a 15 minutes’ period after the
syrup was given. It is difficult to differentiate vomiting and regurgitation as the
reported vomiting occurring immediately after the field worker had left may actually
have been regurgitation. The seemingly increased frequency of vomiting among zinc
recipients in our trials as compared to other studies may, in part, be explained by the
relatively high doses. However, our trials had large sample sizes and thereby sufficient
power to detect excess vomiting. Moreover, being among the predefined outcomes, we monitored vomiting and regurgitation more carefully. The reported zinc-induced vomiting is supported by the findings of a therapeutic trial of acute diarrhea in children in a New Delhi slum (120). In this trial, the children were given an identical formulation and dose as we used in the trial described in paper I. The trial also included a study group that was given ORS with 40 mg elemental zinc (zinc-gluconate) per/L instead of the zinc syrup. Increased risk of vomiting of the same magnitude as we show in paper I was reported among those that were given the zinc syrup compared with those that were given placebo. Children that were given the ORS mixed with zinc, however, did not have an increased risk of vomiting. It should be noted that these children had variable ORS intake and lower mean zinc intake compared with those in the zinc-syrup group. On an average, they consumed 17 mg elemental zinc daily from the ORS, and the effect on the duration of the illness was somewhat less than in the children receiving the zinc syrup. As the three trials described in paper I, III and reference 120 used the same syrup formulation and with zinc-gluconate as the zinc salt, there is a slight possibility that this composition induced more nausea than other formulations that have been used in other trials. However, zinc gluconate is considered to be well tolerated (14) and the placebo syrup only differed from the zinc syrup by lacking zinc gluconate.

We may not have been able do demonstrate an effect on clinical pneumonia in study III using lower zinc doses and the high zinc doses may also have explained the substantial effect on acute diarrhea in study I. This hypothesis that higher doses may be more effective is further strengthened by the fact that in the above mentioned therapeutic trial from India (120), the children that were given zinc syrup had greater effect of zinc than
did the children in the ORS zinc group that consumed on an average less zinc per day.

A more appropriate way of addressing whether there is a dose response, however, is by assessing the effect of various doses of zinc in a clinical trial. In a randomized placebo controlled therapeutic trial in children with acute diarrhea (69), 14 or 40 mg zinc was given to the children in the zinc group. There was only a slightly larger effect of the intervention among those receiving the higher zinc dose. However, this study was not designed to assess a dose-response and a statistical test of dose as an effect modifier was not reported. The optimal doses of zinc for the treatment of acute diarrhea and for preventing clinical pneumonia remain to be decided.

**Animal studies**

In the animal studies, apart from confirming that zinc depletion impedes growth in a dose-response manner (Paper IV), we show that the immune response to PspA was substantially reduced. Furthermore, we demonstrate that zinc depletion results in an increased level of pneumococcal colonization and an increased severity and lethality of pneumococcal infection. The experiment described in paper V was a follow-up of a previous study where we also demonstrated increased severity and lethality of pneumococcal infections in zinc deficient mice (157). To our knowledge, increased severity of pneumococcal infection following zinc depletion has not previously been demonstrated. Immunization with PspA did not significantly reduce lethality but reduced the risk of severe infection, which was also associated with zinc depletion from bones and, among the zinc replete animals, from plasma. The anti-PspA immunity that was present at the time of challenge may have reduced the infection-associated
induction of proinflammatory cytokine responses that in turn induced a redistribution of zinc (61).

Animal studies are useful to demonstrate biological effects. Zinc metabolism and the biological effects of zinc in mice, however, are likely to be different from those in humans. Furthermore, caution is required when comparing the results from the two animal studies as we used somewhat different designs and diets. However, the zinc deficient mice had impaired growth in both experiments and the growth retardation was of the same magnitude.

Despite the effect of zinc depletion on growth, there was no effect of zinc depletion on the response to the pneumococcal polysaccharide antigens (Paper IV). The capsular polysaccharide antigens stimulate the B-lymphocytes directly to produce specific antibodies independently of T-lymphocytes (162). Polysaccharide antigens with repeated saccharide structures as in the capsule of pneumococci, are characterized as type 2 T cell independent antigens (162). Type 2 T cell independent antigens can only activate mature B cells. This response does not require T cell involvement and cellular proliferation and differentiation, but results in specific antibody production from these mature B-lymphocytes. Thus, the T cell dependent and type 2 T cell independent immune responses are different and may thereby face different consequences of zinc depletion. Some animal studies have indicated that the primary effect of zinc deficiency is a reduction in the number of T and B-lymphocytes with little loss of function on the surviving cells (34, 163). If a sufficient number of mature B-lymphocytes are maintained during zinc deficiency and the function is unaltered, the type 2 T cell independent immune response may not be reduced. Other mouse experiments, however,
have shown that type 2 T cell independent responses are substantially, albeit to a lesser
degree than the T cell dependent immune responses, reduced during zinc depletion
(34).

The IgM response mounted against the 23 antigens in the Pneumovax® vaccine is
weaker and varies more than an IgG response to a protein antigen, such as PspA. Thus,
the somewhat small number of animals, higher variability, and the magnitude of the
IgM response may have masked differences in the immune responses between the
experimental groups in paper IV. Detection of a small, statistically significant
difference would require a much larger sample size than we used in our study. The
differences between the four groups, however, were negligible, suggesting that zinc
deficiency does not dramatically affect the immune response to pneumococcal
polysaccharide antigens in mice. The immune response to PspA, which is dependent
on T cell involvement, was substantially impaired confirming that zinc depletion
interferes with T cell dependent immune responses (34).

Accurate estimation of the quantities of food consumed by a mouse is difficult and we
spent a considerable amount of time developing a pair-feeding system with minimal
spillage of fodder. Mice on a zinc deficient diet become increasingly zinc depleted and
will therefore gradually decrease their food intake. We estimated the food intake of the
mice that were offered the diets with the lowest zinc levels over a one (Paper IV) or
two-day (Paper V) period and gave the mice in the other feeding groups an identical
weight-adjusted amount during the next 24 or 48 hours. Our design, however, does not
compensate for this offset in the pair feeding procedure. The zinc depleted mice may
accordingly have had a slightly lower weight-adjusted intake of energy and other
nutrients compared to the pair fed controls. The food intake of the zinc deficient mice, however, was reduced every day for the first week and then stabilized on the same level. Thus, the offset probably influenced the pair feeding procedure, if at all, only during the first week. If the pair feeding was suboptimal, nutrient differences secondary to zinc depletion could have contributed to the observed effects. We believe that the pair feeding was successful and that the observed effects were primarily a consequence of zinc depletion.

In paper V, we assessed the zinc levels in serum and in bones. There were 11 deaths before cardiac puncture could be done and we were therefore not able to analyze serum zinc in these mice. This may have caused a bias in the estimation of the difference in the mean serum zinc concentration between the groups. All fatalities were among the zinc deficient mice, and when we restricted the analysis of the differences in serum zinc levels between PspA immunized and sham immunized mice to those that were offered the zinc adequate diet, the average serum zinc level was substantially (25%) higher in the PspA immunized mice as compared to in the sham immunized mice ($P=0.03$).

Zinc deficient mice had a higher number of pneumococci in their nasopharynx than the zinc replete mice. The above-mentioned fatalities, however, may also have introduced a bias in this analysis. All mice that died before the nasal wash procedure was done were zinc deficient. It is plausible that these zinc deficient mice had even higher numbers of pneumococci in their epipharynxes and we believe that the potential bias is towards the null. Thus, our estimated difference is likely to be conservative. To better address this issue, it may have been preferable to challenge the mice with a less virulent pneumococcal strain. The effect on mucosal carriage, however, may in that case not
have been possible to demonstrate. We could also have avoided severe infections by using a lesser degree of zinc depletion. The latter approach would probably have substantially increased the sample size required to identify effects with adequate precision.
Conclusions and recommendations

The studies in this thesis work show the importance of zinc in common childhood infectious diseases. The field trials in Nepalese and Indian children demonstrated a substantial impact of zinc administration on the severity of diarrhea and on the risk of clinical pneumonia. The effect of zinc on reducing the duration of diarrhea was not dependent on administration of vitamin A and we were not able to demonstrate any effect of therapeutic zinc beyond the enrollment episode. Inflammation severity, as reflected in fever and CRP levels, and plasma zinc concentration were highly correlated. We also found that, in mice, zinc deficiency was associated with impaired growth, increased severity of and increased risk of death from pneumococcal infections and reduced immune response to the pneumococcal protein antigen PspA. Based on our findings, we recommend that:

1) efforts should be made to enhance zinc nutriture in children of developing countries, especially among those with little bioavailable zinc in their diet,

2) because therapeutic zinc reduces the duration of acute diarrhea, it should be incorporated into the standard management of acute diarrhea in children of developing countries,

3) future supplementation trials should assess carefully whether children who are given zinc experience more vomiting than other children,

4) efforts should be made to gather data from completed and planned short-term supplementation trials to reassess the magnitude of any delayed effects of zinc on subsequent morbidity,
5) Plasma albumin concentration and degree of inflammation should be taken into account when interpreting plasma zinc levels in children with common infections such as diarrhea, and

6) Clinical trials on the effect of oral zinc as adjunct therapy in childhood pneumonia with adequate sample sizes and including children with severe pneumonia should be undertaken.
References


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