Zinc, Iron and Infection-studies in children and women in Nepal

Ram Krishna Chandyo

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

2011

Dissertation: October 20, 2011

Collaborations

Centre for International Health, Faculty of Medicine and Dentistry, University of Bergen, Norway

Child Health Research Project, Department of Child Health, Institute of Medicine, Tribhuvan University, Kathmandu, Nepal

Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen, Norway

Department of Epidemiology, States Serum Institute, Copenhagen, Denmark

Department of Community Medicine, Kathmandu Medical College, Sinamanagal, Kathmandu

Siddhi Memorial Children Hospital, Bhaktapur, Nepal

Acknowledgements

The studies included in this thesis were all conducted in Bhaktapur, Nepal in main collaborations with the Department of Child Health, IOM, Kathmandu and Centre for International Health, University of Bergen, Norway. We received grants from the following donors:

1. The European Commission (EU-INCO-DC contract number INCO-FP6-003740)
2. The Research Council of Norway (RCN project numbers 151054 and 172226)
3. The Norwegian Council of Universities' Committee for Development Research and Education (NUFU project numbers PRO 36/2002 and 2007/10177),
4. The Danish Council of Developmental Research (project number 91128),
5. The State Education Loan Fund (Statens lånekasse for utdanning) provided support for me while writing thesis.

The field of research, where I started the very first day of my medical carrier, was completely new for me and was one of the least taught subjects in medical school. But fortunately, I got the opportunity to work in the Norway-India-Nepal consortium on child health research with continuous supervision and scientific guidance from pioneers in global health. This work also provided me with a unique opportunity to work in a field hospital as a clinician as well as a researcher. I would like to express my heartfelt thanks and appreciation to all people in this consortium and specifically to the following individuals who directly or indirectly helped me for a successful completion of my thesis.

Women, Children and their families, who accepted to participate in the studies and trusted our services and the research projects.

Dr. Tor A Strand my supervisor, who since the early stage of my research carrier supervised me and always stressed the importance of good quality

research. His thoughtful experiences and understanding of field research activities, continuous exploring of innovative research ideas, creating optimal environment for team-work, for high quality data management, and for securing grants, were very important for my achievement. Thanks also for your immense contribution by securing support for the field site pediatric hospital for more than 10 years; we all owe sincere gratitude and debt for this help to our community.

Prof. Halvor Sommerfelt, project coordinator of the first study in Bhaktapur for great input on the design, for guidance and for great help in sharpening language of the manuscripts and thesis. Even in your busy time schedule, I could feel your "digital" presence and you were always a great inspiration for me.

Prof. Rune J Ulvik, my co-supervisor for elaborating me thoroughly on body iron metabolism and interpretation of different iron parameters for the iron paper and also contribution to thesis writing.

Dr. Palle Valentiner-Branth, also my co-supervisor, for your time effort in the field and for the help during the manuscript and thesis writing process. Your close supervision at the field site, monitoring daily activities and frequent home visits of enrolled children with field workers even during an unfavorable security situation was indeed fundamental for the success of the clinical trial.

Dr. Maria Mathisen, who stayed in Nepal as a PhD fellow and supervised all activities of the clinical trial. Your tolerance, friendliness and acceptance working in completely new environment and dedicated and close supervision of field and lab staff were crucial for the success of the study and good lessons for us. Thanks also for your input on thesis writing and care during my time in Norway.

Dr. Manjeswori Ulak and Dr. Meeru Gurung, for their contribution in the clinical aspects of the study, recruitment of children throughout the study period

and for help during the writing process. Your involvement and sincere contribution to the community beyond research project was crucial for keeping the unique relationship with the inhabitants of our study area.

Profs. Ramesh K Adhikari, Prakash Sunder Shrestha, Pushpa R Sharma and Associate Prof. Sudha Basnet, at the Department of Child Health, IOM, TU, Nepal for their support and guidance throughout the study. Their distinctive approach to patient care, clinical judgment, constructive feedbacks and teaching will remain as a "Mantra" for my carrier forever.

Field, computer and lab staff for their cooperation and uninterrupted field visits even during unfavorable conditions like raining, wind, cold, festivals or strikes**: Chandrawati Chitrakar, Pasupati Bhakta Ray, Samir KC, Uma Regmi, Biswa Nath Sharma, Govinda Gurung, Bidhya Karmacharya, Bimala Karmacharya, Ram Pyari Rana, Bishnu Maya Kandel, Krishneswori Datheputhe, Shanti Sachin, Sushila Maharjan, Indira Suwal, Shova Bista, Padma Khayargoli, Umesh Tami Shrestha, Shyam Shrestha, Ratna Rajthala, Suvadra Malla, Bandhu Shrestha, Kalpana Neupane, Ashok Dangal, Dipendra Adhikari, Indira Twati, Bina Suwal, Nim Raj Khyaju, Sudan Lama, Shova Pradhan, Sunaina Poudel and Devi Maharjan.**

Shyam Dhaubhadel, founder chairman of Siddhi Memorial Hospital and all hospital staff, for great cooperation, guidance and giving us the opportunity to perform research through the hospital.

Prof. Hemang Dixit, Principal, Kathmandu Medical College for inviting me to join the research consortium which was indeed a turning point of my medical carrier, for the continuous encouragement, and for giving me a unique opportunity to develop my academic carrier.

Mari Manger and Sigrun Henjum, nutritionists and PhD fellows, for sharing knowledge and guidance on dietary recalls as well as friendship and care during my stays in Norway.

Hans Peter Andersen, Department of Geography, UiB, Norway for thoughtful discussions, for sharing ideas on human, agricultural and geographical perspectives of nutrition and for giving me a chance to work in his "Ricebean project".

Trude Fredriksen, for the moral support and hospitality during my visits in Bergen, Voss and Lillehammer and for giving me care like a fourth child in your family.

Administrative and academic staff at Centre for International Health, UiB for providing me optimal and friendly environment to study and for taking care of all logistic requirements efficiently: **Prof. Rune Nilsen, Prof. Gunnar Kvåle, Prof. Thorkild Tylleskar, Prof. Odd Mørkve, Prof. Knut Fylkesnes, Erling, Jamil, Jørn, Sven, Hans, Nils, Borgny, Unni, Solfrid, Linda, Ingvild and Ingunn.**

Foreign students department at UiB for all the support and administrative papers/visa arrangement whenever required, particularly Brit Olaug Kalsnes and Ana Veronica Cordova.

Dr. Viggo Hasseltvedt and **Ingunn L Haavemoen** at the Department of Medical Microbiology of the Innlandet Hospital Trust, Lillehammer, Norway for help with sample preparation and storage and for providing quiet work and living environment for me and my coworkers during our stays in Lillehammer.

Dr. Nita Bhandari and Dr. Sunita Taneja at the Society for Applied Studies, in New Delhi for help during design, formulating research questions and also for the help during my visits to Delhi.

Dr. Yogendra Bhakta Shrestha, senior pediatrician who helped me bring into the medical field and for his continuous guidance and support. It would have been impossible to start my medical carrier without his generous help.

Prof. Indur Dudani, HOD, Community Medicine, Kathmandu Medical College and my colleagues **Dr. Sunil Kumar Joshi, Dr. Abhinav Vaidya a**nd **Umesh Aryal** for their friendliness and great cooperation during my absence and during my hectic schedules with project work.

My family, relatives and well-wisher friends, for their continuous well wishing, blessings for my study and support during my travels and particularly to my father and mother and to my wife **Manju**, for managing the triple burdens of hospital, research and family duties during my travels and to my children **Roman, Rohan and Rojan** for understanding my challenges in the study and excusing me for being away for long time during my study period.

Last but not the least, I also want to acknowledge following remaining staff who were involved in the project in some time point or by supporting administratively or logistically.

Sarita Yakami, Saphalta Shrestha, Rina Hada, Rhinu Shrestha, Kalpana Awal, Ratneswori Prajapati, Radha Prajapati, Prava Prajapati, Dr. Shailendra, Dr. Jarmista, Dr. Rojina, Dr. Adhish, Dr. Anita Gautam, Dr. Anita Pokhrel, Dr. Hem Lata, Dr.Ganendra Raya, Dr. Salma Banu, Dr. Shrawani, Dr. Trishna, Dr. Satya Narayan Suwal, Dr. Samira, Dr. Sumitra Thapa, Babu Ram Neupane, Sama Bhandari, Kedar Bista, Ram Krishna Kuikel and Sukramani Kuikel.

Abbreviations

ALRI	Acute lower respiratory infection
CI	Confidence interval
CRP	C-reactive protein
EAR	Estimated average requirement
HR	Hazard ratio
IMCI	Integrated management of childhood illness
IZiNCG	International zinc nutrition consultative groups
ID	Iron deficiency
IDA	Iron deficiency anemia
LCI	Lower chest indrawing
LBW	Low birth weight
OR	Odds ratio
P:Z	Phytate:Zinc ratio
PSU	Primary sampling unit
p-TfR	Soluble-plasma transferring receptor
RDA	Recommended dietary allowances
RCT	Randomized clinical trial
UNICEF	United Nations Children's Fund
WHO	World Health Organization

Abstract

Malnutrition in the form of underweight, stunting, wasting or micronutrient deficiency is prevalent in developing countries and has serious health consequences, particularly for children and women. Correction of micronutrient deficiencies has been perceived as one of the most cost effective ways to improve maternal and child health and development. Cultural and dietary practices as well as nutrient content of local foods may differ between regions. It is therefore important to describe micronutrient status in representative population samples in developing countries.

Iron and zinc, two essential nutrients which we get mainly from animal source foods, are low in the diet of most of women and children of low and middle income countries because of limited food intake, consumption of a predominantly vegetarian diet and frequent infections. Zinc deficiency is thought to be widespread in these countries and successful replenishment of this single nutrient might reduce morbidity and mortality. Globally, diarrhea and pneumonia together cause almost one third of all deaths in children less than 5 years of age. The beneficial effects of preventive as well as therapeutic zinc supplementation in reducing diarrheal duration and severity are already established. Zinc is now included in the standard treatment protocol for diarrhea management. Clinical trials in low-income countries have also shown that zinc supplementation to healthy children may prevent the occurrence of childhood pneumonia. It is plausible that zinc also may have a therapeutic effect when given during an acute episode of pneumonia. This has been assessed only in a few trials and the results are conflicting. It has also been observed that zinc given for a relatively short period of time (~ 2 weeks) may protect against common

childhood infections for up to 6 months beyond the period of administration. However, the results from the studies examining this “downstream” effect of zinc on prevention of infections are also inconclusive.

We carried out a cross-sectional study in a representative sample of 500 non-pregnant women, 13-35 years of age in Bhaktapur, Nepal. Plasma was analysed for zinc and different biomarkers of iron status. The intake of various nutrients was estimated by dietary recalls. We also conducted a clinical trial where we enrolled 2,628 cases of community acquired pneumonia to measure the efficacy of zinc on the risk of treatment failure, the duration of the enrollment episode and whether or not short-term zinc supplementation reduced the incidence of infections for the subsequent 6 months. In addition to standard antibiotics for pneumonia, children were given zinc sulfate or placebo tablets (age <1 year: 10 mg, ≥1 year: 20 mg of zinc) for 14 days.

From these two large community based studies in Nepalese women and children, we documented that zinc deficiency was very common and coexisted with iron deficiency and anemia. Although, the intake of iron predicted hemoglobin concentration, the intake of zinc was not correlated with plasma zinc concentration. Despite the high prevalence of zinc deficiency and the fact that we have previously demonstrated a beneficial therapeutic effect of zinc on diarrhea in this population, we found that zinc neither reduced the duration of World Health Organization defined pneumonia nor the risk of treatment failure. Nor did short-term zinc administration reduce the burden of diarrheal or respiratory illnesses over 6 months of follow up. Vomiting/regurgitation, which is a well-known side effect of zinc, was more common among children who received zinc than in those who received placebo. Our findings indicate that oral

zinc may not have a role in the treatment of community acquired relatively mild pneumonia in children. Zinc deficiency was common in this community; however, anemia and iron deficiency was substantially less common than available national estimates. Iron deficiency explained only half of the total anemia prevalence suggesting that other causes of anemia may be prevalent in this community.

List of publications

The thesis is based on the following papers:

PAPER I Chandyo RK, Strand TA, Ulvik RJ, Adhikari RK, Ulak M, Dixit H, Sommerfelt H. Prevalence of iron deficiency and anemia among healthy women of reproductive age in Bhaktapur, Nepal. Eur J of Clin Nutr. 2007 Feb;61:262-9

PAPER II Chandyo RK, Strand TA, Mathisen M, Ulak M, Adhikari RK, Bolann BJ, Sommerfelt H. Zinc deficiency is common among healthy women of reproductive age in Bhaktapur, Nepal. J. Nutr. 2009 Mar;139:594-7.

PAPER III Valentiner-Branth P, Shrestha PS, Chandyo RK, Mathisen M, Basnet S, Bhandari N, Adhikari RK, Sommerfelt H and Strand TA. A randomized controlled trial of the effect of zinc as adjuvant therapy in children 2-35 mo of age with severe or non-severe pneumonia in Bhaktapur, Nepal**.** Am J Clin Nutr. Jun 1 2010; 91 (6)

PAPER IV Chandyo RK, Shrestha PS, Valentiner-Branth P, Mathisen M, Basnet S, Ulak M, Adhikari RK, Sommerfelt H and Strand TA. Two Weeks of Zinc Administration to Nepalese Children with Pneumonia Does Not Reduce the Incidence of Pneumonia or Diarrhea During the Next 6 Months. J Nutr. 2010 Sep;140(9):1677-82

Contents

Collaborations……...3

Acknowledgments…….4

Abbreviations……....9

Abstract……....10

List of publications……...……...13

1. Introduction……...17

    1.1. Malnutrition and micronutrient deficiency…….17

    1.2. Anemia and iron deficiency……..……..19

    1.3. Zinc nutrition and role for maternal health…….20

    1.4. Preventive zinc supplementation in children……..21

    1.5. Therapeutic use of zinc for diarrhea……...22

    1.6. Therapeutic use of zinc for pneumonia……..23

    1.7. Short course of zinc supplementation and delayed effect……..27

    1.8. Zinc for childhood mortality reduction……..28

    1.9. Assessment of zinc status in a population……..29

    1.10. Assessment of iron status in a population……..30

    1.11. Interaction between iron and zinc……..……....31

2. Objectives……..32

3. Materials and Methods

3.1. Study site and demography……34

3.2. Food habits……..35

3.3. Study design- Cross sectional study……...36

3.4. Dietary recalls method in the cross-sectional study…….....38

3.5. Dietary reference intakes……38

3.6. Study design- Clinical trial

3.6.1. Enrollment and co-intervention…….39

3.6.2. Follow up procedure for the six months period…….42

3.7. Ethical considerations……42

3.8. Definitions……...43

3.9. Data management and statistical analysis……...44

4. Summary of results

Paper I- Prevalence of iron deficiency and anemia among healthy women of reproductive age in Bhaktapur, Nepal…….46

Paper II- Zinc deficiency is common among healthy women of reproductive age in Bhaktapur, Nepal…….49

Paper III- A randomized controlled trial of the effect of zinc as adjuvant therapy in children 2-35 mo of age with severe or non-severe pneumonia in Bhaktapur, Nepal……50

Paper IV- Two Weeks of Zinc Administration to Nepalese Children with Pneumonia Does Not Reduce the Incidence of Pneumonia or Diarrhea During the Next 6 Months …….....53

5. Discussion

    5.1. Selection and compliance- Cross sectional study……..……..55

    5.2. Selection and compliance – Clinical trial……...56

    5.3. Iron deficiency and anemia…….....56

    5.4. Zinc deficiency……58

    5.5. Intake of phytate……..60

    5.6. Interpretation of nutritive and plasma zinc values……..61

    5.7. Zinc administration for the treatment of community acquired pneumonia……..62

    5.8. Zinc supplementation and delayed effects……64

6. Main conclusions and recommendations…….....67

7. References…….71

8. Papers I-IV

1. Introduction

1.1. Malnutrition and micronutrient deficiency

Malnutrition, which usually is accompanied with deficiencies of individual or several micronutrients, is an important underlying cause of illness and death, particularly in women and children of developing countries [1, 2]. Here, it affects approximately one of every five children and more than half of those who die under the age of five years, are malnourished [3, 4]. Malnutrition leads to increased susceptibility to infectious diseases and contribute to the unacceptably high childhood mortality seen in many low-income countries [5]. Interventions with correction of micronutrient deficiencies have been shown to reduce maternal [6] as well as infant morbidity and mortality [7-9], though some other studies could not replicate these beneficial findings [10-12]. There is a need to identify which nutrients children and women are deficient of, and to find effective and realistic means of dealing with these nutritional disorders.

The underlying causes of malnutrition and micronutrient deficiencies are complex and related to socio-economic status and living circumstances, as described in the United Nations Children's Fund (UNICEF) conceptual framework of malnutrition [13]. Maternal undernutrition reflected as low body mass index (BMI), and stunting in children less than 5 years of age, are two common manifestation of malnutrition. In a recent national survey in Nepal 24% of non-pregnant women of 13-49 years of age were found to have low BMI (< 18.5 kg/m^2) and half of children under five years of age were stunted [14].

Maternal nutritional deficiency is associated with poor pregnancy outcomes [15], low birth weight (LBW) [16] and high growth faltering in children [17]. Women in developing countries may be deficient of several micronutrients before they become pregnant [18, 19]. Information on the prevalence of such deficiencies is to a large extent based on studies from hospitalized patients or from pregnant women. Metabolic changes and physiological hemodilution may influence the serum and plasma concentrations of micronutrient markers during disease and pregnancy. Therefore, laboratory tests may be difficult to interpret. So, sampling of presumably healthy non-pregnant women is important in order to get proper estimates of the micronutrient status [20].

1.2. Anemia and iron deficiency

Iron deficiency (ID) is one of the most common nutritional deficiencies in the world, particularly in developing countries where it affects every other women of reproductive age [21]. Studies from Nepal indicate that as much as two thirds of pregnant women suffer from anemia [22, 23]. Women of reproductive age are particularly vulnerable to anemia and ID due to inadequate intake, multiple pregnancies, recurrent infections and menstrual blood loss. Iron deficiency anemia (IDA), especially during the first 6 months of pregnancy, increases the risk of preterm delivery, LBW, and other pregnancy related complications [24, 25]. IDA during pregnancy has also been associated with poorer cognition and behavior in children as well as poor mother child interaction and emotional attachment during the post–partum period [26].

Daily iron supplementation (60 mg/day) is recommended for all women of reproductive age living in countries where the prevalence of anemia is > 40% [27]. Iron supplementation is also universally recommended during pregnancy and post partum in areas where malnutrition and anemia are prevalent irrespective of the women's body iron status. Similarly, prophylactic iron supplementation is recommended for all children in developing countries where the prevalence of anemia is > 40% [28, 29]. However, caution is warranted in communities where infections are common, as iron supplementation has been associated with increased susceptibility to infections [30, 31] . Although, a study in Southern Nepal [32] did not find any beneficial or adverse effects of iron and folic acid supplementation on mortality, a study in Zanzibar found that those who received iron and folic acid had a 12% (95% CI: 2%, 23%) increased risk of severe illness leading to hospitalization or death and a 16% (95% CI: 2%, 32%) increased risk of adverse effects due to malaria [33]. After publication of this

study, World Health Organization (WHO) issued a statement stressing targeted rather than universal iron supplementation in communities where infection rates or malaria transmission are high, specifically to iron replete children [34]. These findings also emphasize the importance of generating more data on iron status in different regions [35]. This was also one of the conclusions of a recent meta-analysis of iron supplementation which included 26 randomized clinical trials (RCT) in children less than 5 years of age [36].

1.3. Zinc nutrition and its role for maternal health

Zinc is an essential trace element for humans and crucial for normal function of the immune system [37-39], DNA synthesis, cellular division, proliferation and growth [40]. Severe zinc deficiency may lead to hypogonadism, dwarfism and severe growth faltering, increased susceptibility to infections, poor immune responses and chronic diarrhea [41]. Fortunately, now such cases of severe zinc deficiency are uncommon. However, sub-clinical zinc deficiency is widespread globally and probably contributes to the high burden of disease in low income countries [42]. It is estimated that one third of the world's population are at risk of developing zinc deficiency [40]. The low intake of zinc containing food along with the consumption of food containing high levels of phytate, which impairs the absorption of zinc, may be responsible for the high prevalence of zinc deficiency [43]. Moreover, the soil in many developing countries contain only small amounts of zinc and the agricultural products therefore often contain little zinc [44]. To address this wide spread public health problem, WHO/UNICEF/ International Zinc Nutrition Consultative Group (IZiNCG) urged for direct measurement of dietary zinc intake and/or serum zinc estimation in representative populations from different continents, so that intervention programs if required could be carried out without further delay [45].

Zinc is needed during pregnancy for optimal growth and development of the fetus and for maternal tissue expansion [46]. Poor maternal zinc status is associated with negative pregnancy outcomes [47, 48] including spontaneous abortion, congenital malformations, LBW and preterm delivery [49-51]. Although, some studies indicated that prenatal zinc supplementation improved maternal and neonatal zinc status and birth weight, available data are consistent only for reducing the risk of prematurity [52, 53].

1.4. Preventive zinc supplementation in children

Preventive zinc trials during the last 2 decades in children of developing countries consistently showed beneficial effects on prevention of diarrhea and pneumonia [54-56]. A recent meta-analysis, which included 17 clinical trials, concluded that zinc supplementation for at least 3 months reduced the incidence of diarrhea by 14% (95% CI; 7%, 21%), respiratory infections by 8% (95% CI; 1%, 15%), severe forms of diarrheal illness or dysentery by 15% (95% CI; 5%, 25%) and severe forms of respiratory illnesses by 20% (95% CI; 8%, 30%) [55]. Zinc supplementation was also associated with a reduction of the duration of diarrhea and respiratory illness, although the latter was not statistically significant. In one study, routine zinc supplementation reduced incidence of pneumonia when the diagnosis included cough with crepitations or bronchial breathing at chest auscultation, or an episode of acute lower respiratory infection (ALRI) associated with at least one of WHO defined signs of severe illness [57] (OR; 0.74, 95% CI; 0.56, 0.99) but not when the diagnosis was based on elevated respiratory rate or mother's recall (OR; 0.98, 95% CI; 0.86, 1.13). Similar findings of positive effects of zinc supplementation only when using

specific clinical criteria were also found in a subsequent meta-analysis and meta-regression analysis done by Roth *et al*. that included 10 RCTs [56].

A reduction in the incidence of diarrheal and pneumonia illnesses by routine zinc supplementation in children of developing countries could potentially result in a mortality reduction [42] and improvement of weight and height [58]. Indeed, a previous meta-analysis have found a positive effect of routine zinc administration on growth [59], particularly if the child was stunted [60]. However, this was not confirmed by a recent meta-analysis by Ramakrishnan *et al.* that included even more studies [61]. Furthermore, in a recently published large study in India, almost 2,500 children were given either zinc or placebo for four months [62]. The children who were given zinc did not experience enhanced ponderal or linear growth despite having reduced risks of pneumonia and diarrhea.

1.5. Therapeutic use of zinc for diarrhea

Several RCTs in different developing countries have found beneficial effects of therapeutic zinc given during diarrhea in young children [63-65]. Because of this, WHO and UNICEF now recommend oral zinc administration for 10-14 days for the treatment of childhood diarrhea [66]. A meta-analysis conducted after this recommendation also confirmed the beneficial effect of zinc supplementation [67]. After the WHO recommendations, other effectiveness trials have been conducted and found that zinc not only reduced the duration and severity of diarrheal illness but was also associated with increased use of oral rehydration solution in the community [68-70]. Antibiotics and symptomatic treatment are overused and constitute a ubiquitous problem in most developing

countries, irrespective of economic status of the child's family [71]. This problem may be reduced if zinc becomes more commonly used for the treatment of childhood diarrhea [68].

In a subgroup analysis among children with acute diarrhea receiving zinc supplementation, it was revealed that children who had a comparatively more severe infection defined by high fever or C-reactive protein (CRP) concentration benefited more than those who had a milder illness [72]. This finding along with the fact that zinc especially reduced the incidence of lower severe respiratory infections when these were associated severity or with a high CRP concentration [56, 57, 73], rendered us to hypothesize that zinc also could have a therapeutic effect when given during acute childhood pneumonia.

1.6. Therapeutic use of zinc for pneumonia

Respiratory infections are among the main causes of consultation in health centers and for hospital admissions [74], and pneumonia remains one of the leading causes of death in children under the age of 5 years [5]. There has been a dramatic reduction in child morbidity and mortality after introduction of the conjugated vaccines for Haemophilus influenza type B and Streptococcus pneumoniae [75-77]. These two micro-organisms are probably responsible for 70% of hospitalization caused by bacterial pneumonia [78]. Except for respiratory syncytial virus, vaccine development for the other myriad pathogens that cause pneumonia in children seems elusive at least in near future [79]. So, alternative strategies focusing on reducing the burden of respiratory infections should be prioritized.

The available data on the therapeutic effect of zinc in childhood pneumonia range from reduction of pneumonia duration [80], moderate reduction in duration of fever, an effect in boys only, [73] to no effect [81], and even a deleterious effect during the hot season or in those who had high CRP concentration (>40 mg/dL) upon enrollment [82, 83]. All of these studies were based on hospitalized children with severe pneumonia (Table 1) and the efficacy of zinc in non-severe pneumonia – which is much more common, has still not been assessed.

Table 1. Overview of studies evaluating zinc supplementation as an adjuvant therapy on pneumonia in young children

Author/country	Study period	Sample size	Age group	Disease status when zinc was given	Daily dose/duration of supplementation	Main findings: Short-course of zinc resulted in
1. Mahalanabis, D/ India	Not available	85	9mo-15 yr	Severe measles with pneumonia	20 mg of zinc acetate for 6 days	no additional effect
2. Mahalanabis, D/India	1997-98	152	2-24 mo	Hospitalized severe pneumonia	20 mg of zinc acetate for 5 days	significantly reduced duration of fever and very ill status among boys but not in girls
3. Brooks, A/Bangladesh	1999-01	270	2-23 mo	Hospitalized severe pneumonia	20 mg of zinc acetate until discharge	reduced time till recovery and discharge from hospital
4. Bose, A/ India	2003-4	300	2-23 mo	Hospitalized severe pneumonia	20 mg of zinc sulfate until discharge	no effect and longer duration during the hot season or those with elevated CRP
5. Chang, A/Australia	2001-02	215	0-10 yr	Severe ALRI	20 mg of zinc sulfate for infants and 40 mg for older for 5 days	no effect on time to resolution of fever or tachypnoea or duration of hospitalization

Table 2. Overview of studies evaluating short-course zinc supplementation and subsequent morbidities in young children

Author/country	Study period	Sample size	Age group	Disease status when zinc was given	Daily dose/duration of supplementation	Follow up period	Main findings: Short-course of zinc resulted in
1. Roy, SK/ Bangladesh	1987-89	32	3-24 mo	Acute diarrhea	20 mg of zinc acetate for 2 wk	2 mo	length gain, fewer diarrhea and respiratory illness among stunted
2. Roy, SK/ Bangladesh	1987-89	76	3-24 mo	Persistent diarrhea	20 mg of zinc acetate for 2 wk	3 mo	reduction in diarrhea and improved linear growth among underweight but no difference on respiratory morbidity
3. Strand, T/ Nepal	1998-00	1792	6-35 mo	Acute diarrhea	30 mg or 15 mg of zinc gluconate for average 10 days	1 mo	no effect on morbidity
4. Rahman, MM/ Bangladesh	1997-98	800	12-35 mo	Healthy	20 mg of zinc sulfate 2 weeks	6 mo	reduction in diarrhea and dysentery but more ALRI, no effect on growth
5. Walker, CL/ India	2003-05	538	1-5 mo	Acute diarrhea	10 mg of zinc sulfate for 2 weeks	2 mo	no effect on growth and morbidity
6. Bhutta, ZA/ Pakistan*	1993-95	87	6-36 mo	Persistent diarrhea	20 mg of zinc sulfate for 2 weeks	2-3 mo	Not available

* Results of this study only presented in a meta-analysis published in 1999

1.7. Short-course of zinc supplementation and delayed effects

The beneficial effects observed by preventive zinc trials, which require daily or weekly supplementation for several months, might be difficult to achieve outside research settings. The challenges of supplementation programs that require frequent administration for longer duration in resource poor settings are well described elsewhere [84]. Zinc supplementation given for a shorter time period (2 weeks) during acute illnesses was also found to be effective in preventing subsequent illnesses and could be a possible strategy to counteract the negative effect of zinc deficiency. A meta- analysis from 1999 included three short-course zinc supplementation trials and found that the incidence of diarrhea and pneumonia was reduced substantially during the subsequent 2-3 months [54]. Similarly, a preventive positive effect on diarrhea and pneumonia leading to reduction in mortality was observed when short-courses of zinc were given along with oral rehydration solution for the treatment of diarrhea among Bangladeshi children [85]. The incidence of persistent diarrhea and dysentery was decreased but the incidence of ALRI was increased in another trial of short-course zinc supplementation among children residing in urban slums of Dhaka, Bangladesh [86].

The achievement of reductions of morbidity and mortality through short-courses of zinc administration are indeed promising because it is feasible to implement, inexpensive and could be started when visiting health facilities for other illnesses such as diarrhea [87]. However, the available data are mostly based on selected children who were malnourished or just recovering from acute or persistent diarrhea which limits the generalization of these studies (Table 2).

1.8. Zinc for childhood mortality reduction

Sazawal *et al.* demonstrated a 68% (95% CI; 11%-88%) reduction in mortality in full term but small for gestational age infants that were given zinc for 12 months [7]. In this trial, a total of 1,154 infants were followed up and given 5 mg of zinc sulfate or placebo daily. The implementation of treatment protocol for diarrhea with zinc and scaling up zinc program in developing countries may thus have the potential to decrease child morbidity and mortality [42, 88]. Two studies conducted in Bangladesh also demonstrated that zinc administration substantially reduced mortality. In one study, among 1,665 urban poor children aged 2-12 months a weekly high dose (70 mg/day) of zinc given for 12 months reduced mortality by 85% (95% CI; 33%- 97%) [9]. In another study, zinc administration for an average of 7 days to all diarrheal cases was associated with a 51% (95% CI; 6%-75%) mortality reduction [85]. The latter study was a cluster RCT among 8,070 children who were observed for two years. Further, a combined analysis of two recently published large trials on zinc supplementation in Southern Nepal [8] and Zanzibar [12] demonstrated a significant mortality reduction of 18% (95% CI; 4%-30%) in children older than one year of age. However, another large study in 94,359 North Indian children failed to demonstrate a significant effect of long term zinc supplementation [10]. It was speculated that the lack of effect in this trial was due to the fact that everybody also were given iron and folic acid along with zinc or placebo and comparatively lower doses of zinc were used (5 mg for < 6 months and 10 mg for older children). The latter dose of zinc was also tested among 2,052 hospital born LBW children and follow up were done at 3, 6, 9 and 12 months to assess all causes of mortality and diarrheal and respiratory illness [11]. Despite significant

increased plasma zinc concentration among the zinc recipients, this study neither observed any positive effect on morbidity or on mortality.

1.9. Assessment of zinc status in populations

Zinc deficiency is usually not associated with any specific clinical signs. Serum or plasma zinc concentration is the recommended and most widely used marker of zinc status [89]. Several factors such as diurnal variation, serum albumin level, fasting status, recent intake of food, disease status, age, and sex [72, 90] may influence plasma and serum zinc concentration making them unsuitable as markers of zinc status in individuals.

If plasma zinc is not available, the following parameters have been proposed as proxies for the estimation of zinc deficiency by the IZiNCG. 1. Prevalence of stunting in children; although several other nutritional, genetic, and environmental factors might also contribute to stunting in children, it is commonly used as a proxy for the prevalence of zinc deficiency [91]. The WHO considers the prevalence of stunting >20% in a community as a public health indicator and the same cut-off is also used to define that a population is at risk for widespread zinc deficiency. A benefit of using stunting prevalence as a proxy for the occurrence of zinc deficiency is that the data are available for all countries and periodically updated by the WHO (http://www.who.int/nutgrowthdb/en/). 2. National Food Balance Sheets can be used to find the proportion of the population with inadequate intake of zinc based on the amount of bioavailable zinc in national food supplies. 3. Prevalence of anemia – as both iron and zinc are mainly from animal food sources and most of the food that impairs the absorption of iron also affects zinc [92].

1.10. Assessment of iron status in a population

Iron deficiency with or without accompanying anemia in populations can be detected by using several haematological and biochemical iron parameters. The prevalence depends upon body requirements, available resources and the local patterns of nutritional deficiencies. Anemia is defined as hemoglobin (Hb) concentration and/or hematocrit below a specific cut-off value. In contrast to biochemical tests, Hb and hematocrit can be analyzed by a simple device and at a low cost which make these analyses readily available in resource poor field settings [29]. Assessing the iron status by Hb alone, however, is not sufficient since other causes of anemia for instance deficiencies of vitamins A, B9 (folate), B12 and C, may be widespread in the population [93-96]. By analyzing ferritin and soluble plasma transferrin-receptor (p-TfR) in serum or plasma in addition to Hb, one can differentiate between iron depletion due to exhausted iron stores, iron-deficient erythropoiesis and iron deficiency anemia. Although, serum ferritin in general is a valid indicator of depleted iron stores, this test may be less reliable in countries with a high prevalence of infection in the population. This is because serum ferritin is increased by the acute phase response during inflammatory diseases and infections [97]. Thus, in developing countries which in general have a high burden of infectious diseases in the population, it can be difficult to use serum ferritin as the single test to identify iron deficiency. The p-TfR increase quickly when iron stores are depleted [98] and is not affected by infection, age, gender or in pregnancy. However, it may increase due to high cell turnover such as in hemolytic anemia [99]. The ratio of p-TfR to ferritin has also been found to be useful in detecting early iron deficiency [100]. Some other markers of iron metabolism like erythrocyte or zinc protoporphyrin, serum iron, transferrin saturation, total iron binding capacity and red blood cells indices like mean corpuscular volume, proportion of hypochromic red cells, reticulocyte Hb content and mean corpuscular hemoglobin are also used to detect iron deficiency

in the population [101]. One established approach to determine whether anemia is caused by iron deficiency is to give oral iron for 1-2 months. A change in Hb of at least 10 g/L indicates iron deficiency [29]. This procedure, however, is logistically not easy to perform, especially in poor populations where often multiple causes of anemia exist.

1.11. Interaction between iron and zinc

Iron and zinc share the same competitive pathway for absorption [92]. Although, deficiencies of these nutrients are likely to coexist in the same population [102], absorption of either nutrient may be reduced when they are given together as supplements because of competition in the absorption pathways [31, 103, 104]. Intake of high non-heme food impairs the absorption of zinc [105] and a high ratio of zinc to iron, especially in aqueous solution, such as derived from combined iron and zinc supplements, is also found to affect the absorption of iron from the small intestines [106].

2. Objectives of the studies forming the basis for this thesis

1. to assess zinc and iron status in women of reproductive age in Bhaktapur, Nepal,
2. to measure the efficacy of zinc administration during childhood community acquired pneumonia on episode duration, and risk of treatment failure and,
3. to measure the efficacy of zinc administration during childhood community acquired pneumonia on the incidence of common infections for the subsequent six months.

Specific objectives:

2.1 Cross-sectional study- in a representative sample of women of reproductive age in Bhaktapur, Nepal;

Objective 2.1.1: to assess the intake and status of iron using dietary recall and blood test (paper I).

Objective 2.1.2: to assess the intake and status of zinc using dietary recall methods and determination of plasma zinc concentration (paper II).

2.2 Clinical trial

Objective 2.2.1: to measure the efficacy of zinc administration as adjuvant to antibiotics for the treatment of community acquired pneumonia among 2-35 month old children, on the risk for treatment failure and on episode duration (paper III).

Objective 2.2.2: to identify whether or not zinc given for 14 days starting during an episode of pneumonia reduces the risk of subsequent respiratory and diarrheal illnesses over the next 6 months in children in Bhaktapur, Nepal (paper IV)**.**

3. Materials and methods

3.1. Study site and demography

The University of Bergen, Norway in collaboration with the Institute of Medicine, Tribhuvan University, Kathmandu established a maternal and child health research center at Siddhi Memorial Hospital, Bhaktapur, Nepal. Since 1997 several studies on micronutrients and childhood infections have been conducted here [72, 107-109]. The first of these studies was a trial on the efficacy and effectiveness of zinc for adjuvant treatment of acute diarrhea in children [63]. The results from this study contributed importantly when WHO decided to recommend zinc for the treatment of acute diarrhea in children in developing countries [66].

Kathmandu valley includes the districts of Kathmandu, Lalitpur and Bhaktapur. Bhaktapur district has a heterogeneous population; most of the people are farmers, semi-skilled or unskilled laborers and daily wage earners. Bhaktapur municipality, centre of the study site, has a population slightly above 70,000 according to the 2001 census, and most people have agriculture as their main occupation [110]. The study site is located approximately 1,400m above sea level and is densely populated (1,895 people/sq km) [111]. For the cross-sectional study (Paper I and II), we enrolled women residing in the municipality only; in the clinical trial, we also enrolled children from the outskirts of Bhaktapur municipality who came to seek treatment for pneumonia at our hospital.

In and around the Bhaktapur municipality, there are about 50 carpet factories where migrant families live close to and work in for longer or shorter periods. They usually belong to different ethnic groups than the local Newar population and come from various regions of Nepal. The “carpet workers” are becoming a more and more important part of the Bhaktapur population; we therefore ensured that they were adequately represented in the micronutrient survey.

Most of the families in the study area have access to piped drinking water and toilet with central drainage. The vaccine coverage is quite high (>90% for all routine vaccines on national expanded program on immunization schedule) [112] but the proportion who exclusively breastfeed up to 6 months of age is quite low. Based on a survey in the study area, 79% of infants below 6 months of age had already been given semi-solid foods.

3.2. Food habits

The traditional residents of Bhaktapur typically consume foods grown in their own land, while the migrant population relies mainly on foods purchased in the market. Rice and lentils are usually boiled or cooked in pressure or rice cookers, whereas curried vegetables are fried in cooking oil. The eating pattern varies with the season, workload in the field and availability of foods. Eating outside the home, such as in restaurants is not common, particularly among women. However, celebrations of different festivals, particularly among the local residents are common throughout the year. It is estimated that there are about 50-60 festival celebrations per year, during which food with some more variations and larger amount than the usual daily diet is consumed. Generally, main meals of rice with lentils and/or vegetable curry and pickles are consumed twice a day.

Different local green leafy vegetables are widely consumed mainly in the winter and spring season, depending upon the availability. These green leafy vegetables are commonly used dried as an alternative to fresh vegetables during the season when the latter are scarce. It is common to consume rice flakes or home-made breads from unrefined wheat flour as a mid day snack.

3.3. Study design - Cross-sectional part (objectives 2.1.1 and 2.1.2)

We used a cluster stratified random sampling procedure for the studies presented in paper I and II. We defined two strata, one stratum of local resident women and the other of those residing in the carpet factories within the Bhaktapur municipality. There are 17 administrative units called *Wadas* and about 128 neighbourhoods called *Toles* in the municipality. We used *Toles* and the carpet factories as the primary sampling units (PSU). A total of 23 *Toles* and 5 carpet factories were randomly selected as PSUs.

Prior to enrollment, we obtained a list of all 2,736 women between 13-35 years living in these selected clusters. The enrollment took place from September 2000 to November 2001. Seven hundred and ninety-two randomly selected women were thus selected and approached. We tried to ensure that all women in the sampling frame had the same probability of being included in the study. Two hundred and ninety-two women whom we approached could not be enrolled, mainly because they had moved or did not have time to come to the study clinic for enrollment. Most of the women that had moved away were from the carpet factory stratum (133 women). Table 3 summarizes the characteristics of those that were included in the studies that comprise this thesis.

Table 3. Outline of studies included in thesis

	Cross-sectional Study	**Randomized clinical trial**
Objectives	To estimate prevalence of iron and zinc deficiency and identify factors associated with it	To estimate the efficacy of zinc as adjuvant therapy during community acquired pneumonia and morbidity during subsequent 6 months of follow up
Sample size	500	2,628
Disease status	Healthy	With community acquired pneumonia
Age group	13-35 years	2-35 months
Study area	Bhaktapur municipality	Bhaktapur municipality and outskirt communities
Selection/ randomization procedure	Random sample, cluster, stratified	Double blind, stratified block randomized
Inclusion criteria	Healthy non-pregnant women	Children with community acquired pneumonia and not planning to move from study area for at least for 6 months
Exclusion criteria	Women taking vitamins, minerals, known case of chronic disease or suffering from acute illness	Very severe diseases requiring prompt referral to hospital, dysentery, cough more than 14 days, congenital heart disease, tuberculosis, severe malnutrition or use of antibiotic within last 48 hours
Intervention	Not applicable	Zinc or placebo tablets dispersed in water or breast milk for 14 days along with standard antibiotics. 10 mg elemental zinc to infants and 20 mg to children over 1 year of age
Follow up	Not applicable	Daily for the first 2 weeks then passive follow up for 6 months
Main outcome	Proportion with iron and zinc deficiency and factors associated with zinc and iron	Duration of episode illness, proportion with treatment failure and illness during 6 months of follow up
Publications	In Paper I and II	In Paper III and IV

3.4. Dietary recalls method in the cross-sectional study

In a sub-sample of 394 women, we administered two 24-hour dietary recalls approximately one week apart. The women were asked to recall the foods they consumed the previous day from waking up until going to bed. The first recall was carried out in the clinic, whereas for the second recall, the field workers visited each participating woman in her home. Models of commonly used local foods were shown during the interviews to estimate the portion sizes. Both recalls were completed by the subgroup of 379 women. We used the mean intake of the two recalls in the analysis.

At enrollment, we also administered a semi quantitative food frequency questionnaire (FFQ), using a list of 53 locally consumed food items developed during a previous survey. The frequency of consumption was categorized into never, 1-5 times in the past 6 months, 1-3 times every month, once a week, 2-4 times a week and once or more per day.

3.5. Dietary Reference Intakes

Recommended dietary allowance (RDA) and estimated average requirement (EAR) are two dietary reference indicators, which are commonly used to compare adequacy of nutrient intake. We used the Institute of Medicine, National Academy of Science (NAS), U.S., cut-offs for RDA and EAR for zinc and iron intake [113]. EAR is the average daily nutrient intake, which is estimated to meet the requirement of 50% of healthy individuals of a specific life stage and gender. The EAR cut-off values are used to estimate the prevalence of inadequate intake in groups or individuals. RDA is set two standard deviations above the EAR, and is the intake that is sufficient to meet the nutrient

requirement of nearly all (97.5 %) of healthy individuals. Thus, individuals having an intake at or above this level have a low probability of being deficient.

3.6. Study design - Clinical trial

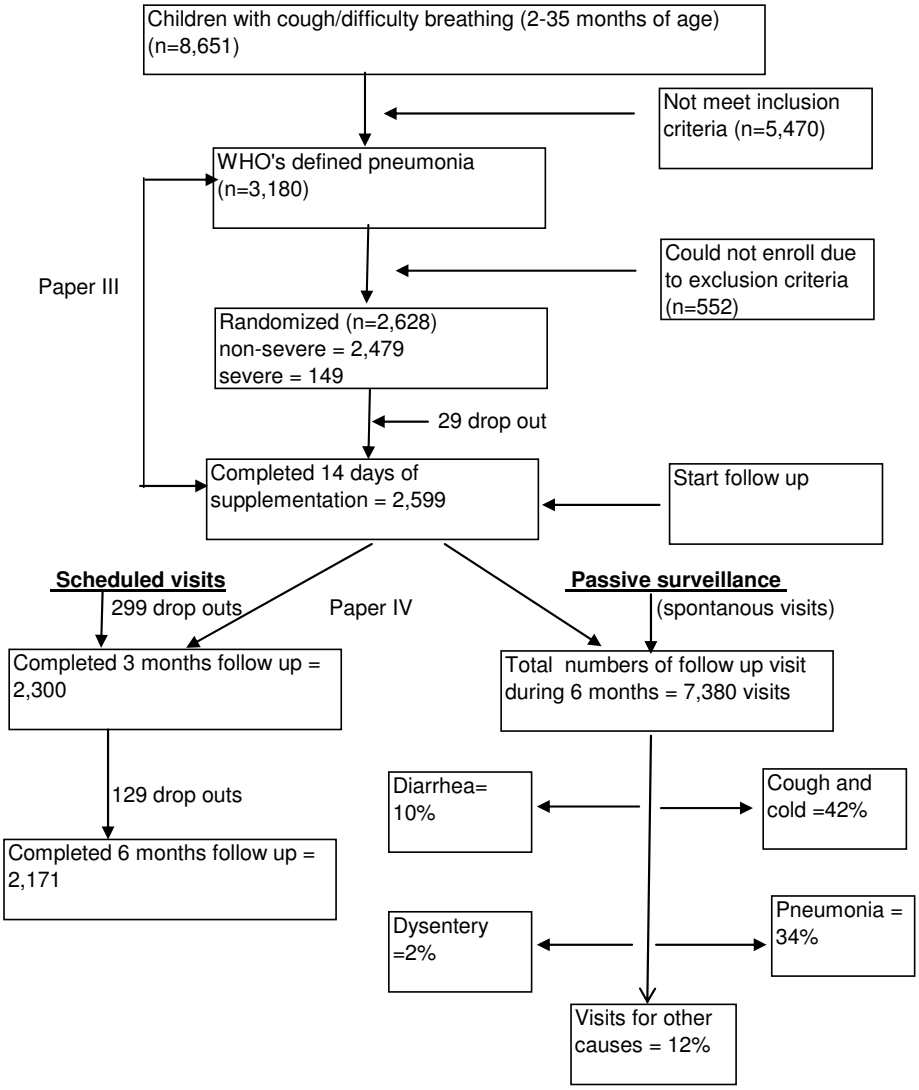
3.6.1. Enrollment and co-intervention

Eligible children were recruited from Siddhi Memorial Hospital, where children were brought for consultation spontaneously or referred by one of the study field workers visiting at home in regular interval. Children with cough or difficulty breathing were screened and enrolled if informed consent was obtained from one of the child's caregivers, inclusion criteria were met, and exclusion criteria were not. The inclusion criteria were 2-35 months of age with non-severe or severe pneumonia as defined in the Integrated Management of Childhood Illness (IMCI) WHO manuals [114] and planning to stay in the study area for at least 6 months. The exclusion criteria are presented in Table 3. Following stratification, according to severity of pneumonia and by age below or above one year, we randomized children in blocks of 16. Dispersible zinc or placebo tablets that were produced in France (Nutriset, Malaunay, France) were dissolved in water or breast milk and given daily for two weeks. The zinc tablets contained 10 mg of elemental zinc sulfate; children less than one year of age were given one and older children were given two tablets. Morbidity visits were done daily until recovery, which was defined as the first of two consecutive days with normal respiratory rate.

Standard antibiotics (cotrimoxazole for 5 days as the first line antibiotic for non-severe and intravenous benzyl penicillin for severe pneumonia) were given to the trial participants. Oral cotrimoxazole was changed to amoxycillin by the

study physician if respiratory rate was still high 72 hours after enrollment. The study physicians changed the antibiotic regimen before 72 hours if the general condition of the child deteriorated or the child required hospital admission. For children with severe pneumonia, injected chloramphenicol was used as the second line antibiotic if lower chest indrawing (LCI) persisted after 48 hours of hospitalization. Children with wheezing were given 2 doses of 2.5 mg of nebulized salbutamol 15 minutes apart and reassessment was done after 30 minutes. The approach and treatment of childhood illnesses was based on IMCI guidelines. Figure 1 shows the details of enrollment and the follow up process.

Figure 1. Enrollment and follow up process of clinical trial evaluating efficacy of zinc supplementation on childhood pneumonia and delayed effects



3.6.2. Follow up procedure for the six months period

We followed the children for 6 months after enrollment. The caretakers were requested to bring their child for consultation whenever they felt it was necessary. Each visit was recorded by one of the study physicians by filling in a morbidity questionnaire after the clinical examination. Additionally, scheduled follow up visits were done at 3 and 6 months after enrollment at the field hospital. During these visits, the study physician obtained morbidity and hospitalization information for the preceding 2½ and 3 months, respectively.

3.7. Ethical considerations

The institutional review board at the Institute of Medicine, Tribhuvan University, Kathmandu, Nepal Health Research Council, Kathamdnu and the Regional Committee for Medical and Health Research Ethics (REK VEST), Norway approved both studies. The implementation of all aspects of the project was in agreement with the International Ethical Guidelines for Research Involving Human subjects as stated in the latest version of the Helsinki Declaration. Iron and deworming medicines were given to all enrolled subjects with anemia, according to national guidelines. Participation was voluntary; the participants could withdraw consent without giving reason at any time. All women and children were offered examination by a physician when indicated. Project card, which entitled a child to have free check up at the study hospital, were provided to all surveyed children irrespective of whether they were enrolled or not. Additionally, we provided 24-hour emergency and in-patient services at our study hospital to all children during the study period. An interim analysis by a data safety and monitoring board was carried out after

approximately one third of the children were enrolled in the clinical trial. The conclusion of this analysis was to continue the study without alterations to the protocol and to achieve the planned sample size.

3.8. Definitions

In the cross-sectional study, anemia among women was defined as Hb concentration < 12 g/dL. We also presented the anemia prevalence after adjusting for the altitude of Kathmandu [115]. Depleted iron stores was defined as plasma ferritin < 15 µg/L [116], and iron deficient erythropoiesis was defined as normal Hb with depleted iron stores and an increased p-TfR > 1.54 mg/L. IDA was defined as depleted iron stores and elevated p-TfR concurrent with anemia (Paper I).

The cut-offs for defining zinc deficiency are different according to the time of blood collection and age group [117, 118]. The EAR of zinc using an unrefined plant based diet is 9 mg/day for women aged 13-18 y and 7 mg/day for non-pregnant women older than 18 years [45]. The EAR of iron for non-pregnant women of reproductive age is 8.1 mg/day. Women consuming less than these cut-offs were considered to have inadequate intake (Paper I and II).

In the clinical trial, pneumonia was defined according to the WHO/IMCI guidelines [114] i.e. history of cough or difficulty breathing combined with elevated respiratory rate i.e. ($\geq$40 breaths/minute for children $\geq$12 months and $\geq$50 breaths/minute for children 2-11 months of age) [114]. A child with cough and difficulty breathing and LCI was considered as having severe pneumonia.

Respiratory rates were counted twice and if required, even more times if the difference between the first and second count was more than 10. Whenever possible, we counted respiratory rates when the child was awake and quite/calm as it may increase during crying or agitation and decrease when the child is sleeping. Diarrhea was defined as the passage of three or more watery or loose motions in the last 24 hours with a recent change in stool character. Dysentery was defined as a diarrhea with a history of blood- mixed stool.

3.9. Data management and statistical analysis

The field supervisors manually checked all forms before they were sent to the computer facility for data entry. The data was double entered into Microsoft Visual FoxPro databases with computerized logic, range and consistency checks. The daily intake of various nutrients was calculated using Indian food tables from the Wfood2 software program [119]. The total zinc and iron contribution by different foods were derived from the nutritive values of the 24-hour dietary recalls, whereas the frequency of reported consumption was derived from the FFQs. The molar ratio of phytate to zinc (P:Z ratio) was calculated by dividing the millimoles of phytate intake (phytate intake in mg/molecular weight of phytate i.e. 660) by the millimoles of zinc intake (zinc intake in mg/molecular weight of zinc i.e. 65.4) [92].

Multiple linear regression analyses were used to determine the associations between Hb with other relevant variables (paper I). The generalized additive model derived graphs were generated using the statistical software R version 1.9.0 to describe the relationships between Hb with p-TfR and iron intake (paper I) and plasma zinc with albumin concentration (paper II). We used the survey

(svy) commands in Stata to adjust the standard errors for the stratified cluster design in paper I and II.

We compared the time until recovery from pneumonia and length of hospital stay between the treatment groups by Cox proportional hazards models. For the outcome treatment failure, we used logistic regression models (paper III). We coded the outcomes and interventions so that hazard ratios (HR) <1 and odds ratio (OR) >1 would represent beneficial effects of zinc administration.

We used 6 months as the follow up period in the morbidity analysis for the children who were available both at 3 and 6 months after enrollment (paper IV) and the time until the first episode of diarrhea and pneumonia between the study groups were compared by Cox proportional hazard regression analyses. For children who had incomplete follow up, the last contact at the study hospital based on a scheduled (monthly-surveillance or 3- monthly) or spontaneous visit was used to calculate the total follow up time. If more than two months lapsed between any contact with the child and our study team, the child was censored on the day of his or her last encounter before this gap of follow up. The incidence rates for pneumonia and diarrheal illness were calculated by dividing the total number of a particular illness by the follow up days contributed by each child in the study and then multiplying this number by 30.42 to obtain a monthly rate. The analyses were undertaken using Stata (STATA Corp, Houston, TX) and in paper III and IV adjusted for multiple entries of the same child by the cluster option in the regression models.

4. Summary of results

PAPER I

Prevalence of iron deficiency and anemia among healthy women of reproductive age in Bhaktapur, Nepal

In this cross-sectional study of 500 women of reproductive age in Bhaktapur, Nepal, we aimed to determine the prevalence of anemia and iron status assessed by biochemical markers and to explore the associations between markers of iron status and iron intake. The definitions and different stages of iron deficiency and percentile values of iron indices are shown in **Table 4 and 5**. The prevalence of anemia (hemoglobin concentration <12 g/dL) was 12% (n=58). The prevalence of depleted iron stores (plasma ferritin <15 μg/L) was 20% (n=98), whereas the prevalence of iron deficiency anemia (anemia, depleted iron stores with elevated transferrin receptor i.e. >1.54 mg/L) was 6% (n=30). Seven percent (n=35) of women had iron deficient erythropoiesis (depleted iron stores and elevated transferrin receptor but normal hemoglobin). Out of the 58 anemic women, 41 (71%) also had elevated plasma transferrin receptor and 31 women (53%) also had depleted iron stores. Fifty-four percent of the women ate less than the recommended estimated average intake of iron. The main foods contributing to dietary iron were rice, wheat flour and green and dry vegetables.

Table 4. Stages of iron balance and its prevalence figures among 500 non-pregnant women of Bhaktapur, Nepal

Stages	**Hemoglobin, (g/dL)**	**Plasma transferring receptor, (mg/L)**	**Plasma ferritin, (μg/L)**	**Prevalence, n (%)**
Normal iron status	≥ 12.0	≤ 1.54	> 15	264 (53%)
Depleted iron stores			< 15	98 (20%)
Iron deficient erythropoiesis	≥ 12.0	> 1.54	< 15	35 (7%)
Iron deficiency anemia	< 12.0	> 1.54	< 15	30 (6%)
Anemia of chronic disease	< 12	≤ 1.54	> 50	5 (1%)
Anemia*	< 12.0			58 (12%)

* 41 (71%) women of those with anemia also had elevated plasma transferrin receptor, whereas 31 (53%) had depleted iron stores.

Table 5. Mean, median and interquartile ranges of zinc and iron parameters among children and women in Bhaktapur, Nepal

Biochemical markers	N	Mean	SD	25th centile	Median	75th centile
Cross-sectional study participants (Among non-pregnant women 13-18 years)						
Hemoglobin, g/dL	136	12.8	1.5	12.3	13.1	13.8
Ferritin, µg/L	136	26.9	35.6	12	23	33.6
p-TfR, mg/L	136	1.8	1.3	1.2	1.4	1.9
Plasma iron, µmol/L	136	12.8	5.7	8.7	12.3	16.8
Zinc, µmol/L	136	8.5	2.1	7.1	8.4	9.4
Among non-pregnant women (≥ 19 years)						
Hemoglobin, g/dL	364	13.4	1.2	12.8	13.5	14.1
Ferritin, µg/L	363	38.3	27.5	19	33	52
p-TfR, mg/L	363	1.6	0.6	1.2	1.4	1.7
Plasma iron, µmol/L	363	14.8	5.7	10.8	14.7	18.6
Zinc, µmol/L	362	8.5	2.6	7.2	8.3	9.5
Clinical trial participants- baseline values- children 2-5 months of age						
Hemoglobin, g/dL	562	10.9	1.0	10.1	10.9	11.6
Zinc, µmol/L	94	8.7	2.7	6.9	8.3	10.2
6 months of age or above						
Hemoglobin, g/dL	2066	11.2	1.2	10.4	11.2	12
Zinc, µmol/L	323	8.9	2.7	7.3	8.6	10.3

PAPER II

Zinc deficiency is common among healthy women of reproductive age in Bhaktapur, Nepal

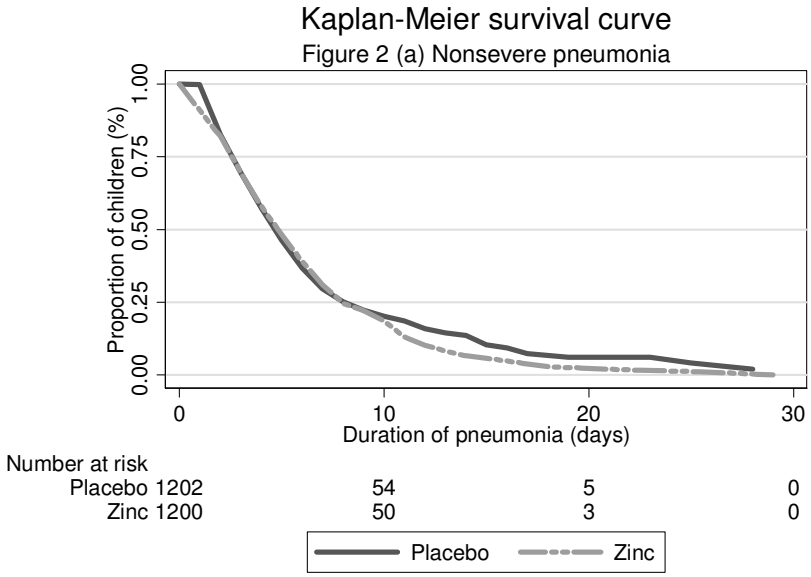
We enrolled 500 non pregnant women in a dietary survey. The objective of this study was to estimate the proportion of women with zinc deficiency and describe its relation with biochemical and other dietary intake factors. The mean plasma zinc concentration was 8.5 μmol/L and the prevalence of zinc deficiency was 78%-90% depending on the definition used. The interquartile range of zinc intake was 7.2-9.4 mg and prevalence of inadequate zinc intake was higher in women ≤ 18 years of age (69%) than in those that were older (29%). The mean phytate intake was 2,198 mg/day with a Phytate:Zinc molar ratio of 26:1. The intake of zinc was strongly and positively correlated with iron intake ($r = 0.79$, $P = <0.001$) and predicted Hb concentration. The plasma zinc concentration was negatively associated with phytate intake ($r = -0.15$, $P = 0.003$) but not with the intake of zinc.

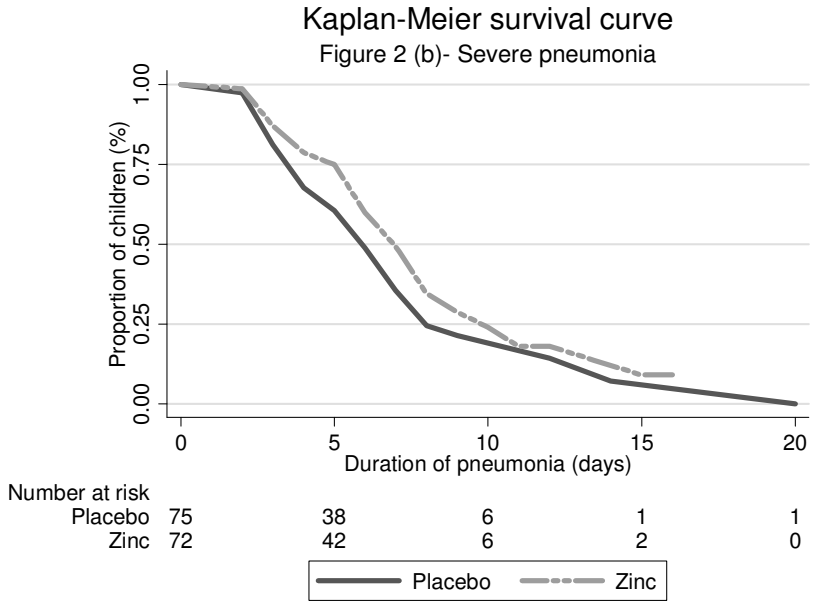
PAPER III

A randomized controlled trial of the effect of zinc as adjuvant therapy in children 2-35 months of age with severe or non-severe pneumonia in Bhaktapur, Nepal.

To measure the efficacy of zinc administration as adjuvant therapy on duration and severity of community acquired pneumonia, we undertook a randomized clinical trial among Nepalese children 2-35 months of age. Zinc was given for 14 days in a daily dose of 10 mg elemental zinc for those less than one year of age and 20 mg for older children along with standard antibiotics according to the WHO Integrated Management of Childhood Illness guidelines. A total of 2,628 episodes of pneumonia cases were enrolled from January 2004 to June 2007. Most of the cases (94%) were non-severe pneumonia. There was no difference in time to recovery between the study groups [hazard ratio (1.0, 95% CI: 0.96-1.1 for non-severe and 1.1, 95% CI: 0.79, 1.5 for severe)]. The mean duration of time till recovery among those with non-severe pneumonia was 3.0 days (SD 2.8) in the zinc group and 2.9 days (SD 2.9) in the placebo group. The corresponding figures in the severe pneumonia stratum were 5.5 (5.6) and 4.6 (3.1) days, respectively. Thirty- nine percent of children with non-severe pneumonia in the placebo and 36% in the zinc group did not have any days of elevated respiratory rate after enrollment. The survival curves of day wise duration of non-severe and severe pneumonia episode is presented in Figure 2. Nineteen percent of the children required antibiotic change and there was no difference in the risk of such treatment failure (OR=0.95, 95% CI: 0.78, 1.2 for non-severe and 0.97, 95% CI: 0.42, 2.2 for severe pneumonia) between zinc and placebo recipients. Regurgitation within 15 minutes after administration of the first dose was observed in 14% of the participants in the zinc group and in 3.8% of placebo recipients (OR=3.9, 95% CI: 1.2-5.5).

Figure 2: Kaplan Meier survival curves of non-severe (Figure. 2a) and severe pneumonia (Figure. 2b) among 2-35 months old children in Bhaktapur, Nepal




Kaplan-Meier survival curve
Figure 2 (b)- Severe pneumonia
Proportion of children (%)
0.00
0.25
0.50
0.75
1.00
0
5
10
15
20
Duration of pneumonia (days)
Number at risk
Placebo 75 38 6 1 1
Zinc 72 42 6 2 0
Placebo
Zinc

PAPER IV

Two weeks of zinc administration to Nepalese children with pneumonia does not reduce the incidence of pneumonia or diarrhea during the next 6 months

We measured whether or not 14 days of zinc administration given to children presenting with community acquired pneumonia prevented diarrhea and respiratory tract infections for another six months. Children enrolled in the trial described in PAPER III were followed for up to six months after enrollment. These children visited the clinic 7,380 times for consultations with the study doctors. Most of these visits were for cough or cold and followed by pneumonia or diarrhea. Every second child from both groups visited at least once for pneumonia and one in five made a visit for diarrhea**.** The median numbers of days until the first episode of pneumonia and diarrhea were similar in the two study groups. The hazard ratios (95% CI) were 1.02 (95% CI: 0.92, 1.14) for non-severe pneumonia, 1.11 (95% CI; 0.72, 1.73) for severe pneumonia, 1.07, (95% CI; 0.91, 1.26) for diarrhea, and 0.96 (95% CI; 0.69, 1.34) for dysentery. The total numbers of visits for these illnesses were also similar in both trial arms. The plasma zinc concentration was significantly higher at the end of administration (9.2 µmol/L in the placebo and 14.6 µmol/L in the zinc group, P= <0.0001) among those who received zinc. However, the plasma zinc concentration was not significantly different between the study groups on blood samples taken on day 45^{th} or 90^{th} after enrollment (Table 6).

Table 6. Plasma zinc status by treatment groups at baseline, and 14, 45 and 90 days after enrollment in zinc intervention trial for pneumonia in Nepal

Variable	N	Mean	Std. Dev.	Mean changes	% with <9.9 μmol/L	Min	Max
Baseline							
Placebo	211	8.8	2.3		70.1%	2.7	15.8
Zinc	206	8.9	2.9		70.3%	2.5	35.6
P-value		0.5					
14 days							
Placebo	89	9.26	2.5	0.5 (3.1)	71.1%	2.5	22.9
Zinc	88	14.62	7.3	5.8 (6.7)	24.7%	7.3	51.7
P-value		<0.005		<0.005			
45 days							
Placebo	38	9.7	2.3	0.1 (2.3)	56.4%	4.8	12.7
Zinc	43	9.8	2.2	1.2 (3.2)	44.4%	5.7	16.1
P-value		0.9		0.1			
90 days							
Placebo	41	9.0	2.3	0.6 (2.5)	70.7%	3.9	15.3
Zinc	39	9.9	3.3	0.8 (3.8)	55.0%	4.6	26.3
P-value		0.1		0.7			

5. Discussion

In these studies, we have estimated the status and intake of two important micronutrients (iron and zinc) in women of reproductive age and measured the efficacy of zinc for the treatment and prevention of community acquired pneumonia in young children. Zinc deficiency was much more common than was iron deficiency and affected approximately four of five women, whereas iron deficiency was found among one in five women. The intake of iron was positively associated with Hb concentration, but the intake of zinc was not associated with plasma zinc concentration. Phytate intake contributed importantly to a high P:Z molar ratio in many women's diet and this ratio was negatively associated with their plasma zinc concentration. Two weeks of zinc administration during severe or non-severe community acquired pneumonia among the trial children in Nepal did not reduce the severity or duration of the illness. The incidence of pneumonia and diarrhea during the 6 months follow up period of these children was also similar in the placebo and intervention groups.

5.1. Selection and compliance –Cross-sectional study

For the cross-sectional part of the PhD project (paper I and paper II), within each stratum of local residents or those working in carpet factories, we randomly selected clusters. The stratum with local resident women constituted 80% of the total sample and the sampling procedure went smoothly throughout the study. However, in the stratum consisting of women from carpet factories there was frequent migration and we had to update the list of eligible women after the study start. The local resident women belonged primarily to the Newar ethnic group and were living in a semi-urban area. Whereas women in carpet factories usually had migrated from other parts of Nepal and mostly belonged to Tamang and Magar ethnic group. Thus, the findings presented in this study may have been somewhat different if studied in other ethnic

groups of Nepal. However, by deliberately recruiting women in carpet factory, other ethnic groups were also to some extent represented.

5.2. Selection and compliance -Clinical trial

For the clinical trial, children were recruited through spontaneous visits to the study hospital or by identification of sick children by field workers through a continuous surveillance of the population (paper III and IV). A total of 98.6% of enrolled children completed the prescribed course of zinc or placebo for 14 days and 85% of them were available at the 6 months follow up visit, which reflect good compliance. The home visits of field workers were checked independently by supervisors in about 4% of the total visits.

5.3. Iron deficiency and anemia

In a representative sample of 500 non-pregnant women, we found an anemia prevalence of 12% which is lower than what was estimated in the nationwide 1998 survey which showed a prevalence of 68% [120]. More recent data from the national demographic health survey estimated a substantial lower anemia prevalence but this figure was still three fold higher than what we found [14]. Most of the women with anemia had mild or moderate anemia but were altered erythropoiesis and depleted iron stores. As pointed out by Guyatt *et al.* [121] iron stores in our body start to decrease already at a ferritin concentrations of 30 – 40 µg/L. Also, living at a high altitude (i.e.1400 m above sea level) may increase the demand of iron for erythropoiesis.

Compared with older women, adolescent girls, 13-18 years of age, had about twice as high prevalence of anemia and iron deficiency. This indicates that this group is at a higher risk, probably because of inadequate intake, growth spurt and/or hormonal changes. Although, anemia is considered a huge public health problem in Nepal, most of the data is based on only estimation of Hb concentration. The etiology of anemia has not been extensively studied, however, and it is assumed that most cases are caused by iron deficiency. The finding of comparatively low Hb concentration among women from carpet factories was not unexpected. The women from the carpet factories have in general lower socio-economic background and had migrated from other parts of Nepal, and do not usually have their own home and are often without support from a joint family.

Our study is, to our knowledge, the first to assess anemia and iron deficiency using multiple iron parameters from a representative sample of healthy non-pregnant women in Nepal. The relatively low prevalence of anemia and iron deficiency in our study could be due to the fact that we enrolled apparently healthy women and from an area that is not endemic for malaria, hookworm infestation or hemoglobinopathies. Furthermore, we found positive associations of Hb with age, parity and use of Depo-Provera (depot medroxyprogesterone acetate). Nearly half of the women reported using Depo-Provera which may contribute to the relatively high Hb concentration. This could be due to cessation of menstruation after Depo-Provera and also enrichment of iron stores from iron supplementation during pregnancy and lactation.

Inadequate intake of iron was observed in more than half of the women and the intake of iron was positively and linearly associated with Hb concentration when the intake was less than the EAR (paper I). Meat contributed only 5% of the total iron intake, indicating that most of the consumed iron was from non-heme food sources. The

absorption of iron is mainly controlled by the iron requirements of the body and affected by several enhancers and inhibitors in foods.

The findings that a high proportion of women had anemia without iron deficiency needs further clarification. Although, iron is a main cause of anemia, deficiency of other nutrients like folate, vitamin B12, vitamin A and zinc may also cause anemia [96, 122, 123]. The prevalence of cobalamin deficiency based on low plasma cobalamin and high plasma homocystein and methylmalonic concentration among pregnant [95] and non-pregnant [124] women of Nepal, has been reported before. We also measured the cobalamin and folate status in this sample of women and we found that cobalamin deficiency was common. However, neither folate nor cobalamin was associated with hemoglobin concentration or anemia [124].

5.4. Zinc deficiency

We found a high prevalence of zinc deficiency affecting more than three fourths of the non-pregnant women. Most of women with anemia (93%) also had zinc deficiency. Plasma zinc concentration is considered the best method for estimating the prevalence of zinc deficiency in populations, if sufficient precautions are taken during sampling and processing and interpretation [89]. Public health intervention strategies including dietary diversification and food fortification, are recommended, if the prevalence of zinc deficiency in a given population is more than 20% [125]. Based on the stunting prevalence in children < 5 years of age, which is also used as a proxy for the estimation of zinc deficiency, Nepal belongs to a category of countries with severe zinc deficiency [117]. Our findings support this assessment but it is unclear whether the stunting prevalence of children under 5 years of age can be used for estimating the prevalence of zinc deficiency in women. Our findings of high

prevalence of zinc deficiency is also supported by other studies in Nepal [19], India [126] and other developing countries [127, 128]. The intake of zinc was not associated with plasma zinc concentration in our study. The intake of phytate, however, was negatively associated with the plasma zinc concentration. The positive association of zinc intake with intake of iron and hemoglobin concentration is probably explained by the fact that both of the minerals are present in same food sources. This may also explain our finding of a positive association of plasma zinc with Hb, which is in concordance with findings in other studies [122, 129]. However, it is noteworthy that zinc deficiency in this population is four fold-higher than that of iron deficiency. We identified variables that were positively associated with the Hb concentration in this population such as age, parity and the use of Depo-Provera contraceptive injection but none of these factors were associated with plasma zinc concentration (data not shown). A recent study of pregnant women in Ethiopia also found high prevalence of zinc deficiency and low prevalence of ID [128].

Although, plasma zinc did not differ by age, the prevalence of inadequate zinc intake was found to be twice as high among those younger than 18 years of age compared to those who were older. This could be because of the higher EAR for the younger women which is 2 mg higher. The findings that the zinc deficiency prevalence was even higher than what would be expected from the proportion of inadequate zinc intake could be explained by the fact that high dietary phytate reduced the bioavailability of zinc. This explanation is supported by our finding that phytate, but not zinc intake was associated with the plasma zinc concentration.

Because of its crucial role in maintaining immunity, growth and cell division [39], and in prevention of common childhood infections, zinc deficiency is estimated to be responsible for 4.4% of all deaths among children 5 years of age globally [42]. Zinc is

also required during pregnancy for foetal growth and for maternal tissue accretion [130]. Observational studies have shown that poor zinc status is associated with negative pregnancy outcomes [46], reduced birth weight [47] and congenital anomalies. The effect of zinc supplementation during pregnancy has also been investigated in several clinical trials in developing countries. A recent meta-analysis showed a 14% and statistically significant reduction in the risk of prematurity which may again have impact on neonatal and infant mortality. However, there were no substantial or significant effects on other outcomes, such as birth weight, neonatal mortality, post-natal morbidity, birth asphyxia, hypertension, or eclampsia in the mother [52].

Apart from age, Hotz *et al.* suggested that gender; time of blood collection and fasting status should be taken into account when interpreting plasma zinc status [131]. In our study, the mean plasma zinc concentration did not vary with time of the day of blood collection. This may be because we collected blood only between 9 AM and 3 PM. The mean zinc concentrations in fasting specimens or specimens drawn from women who had not had a morning meal were substantially higher than in non-fasting specimens, which are in concordance with other findings [132, 133]. However, it is interesting to note that this difference was observed when the women had had their last meal more than 2 hours earlier. It suggests that overnight fasting, as found in a study by Wallock *et al.* [134], or fasting for longer time periods results in increased plasma zinc concentration, whereas fasting for shorter time periods (2-3 hours) does not change plasma zinc concentration much.

5.5. Intake of phytate

Phytate, which is abundant in plant based foods like legumes, nuts, and cereal grains, is an organic compound which binds to minerals like zinc and iron and therefore

interfere with the absorption of these metals in the gastrointestinal tract [135]. Several factors relating to food processing (such as storage, milling, cooking) as well as the variety of dietary components in food composition tables may affect the interpretation of phytate intake of individuals or groups. However, phytates was found to be degraded only marginally in a study measuring the degree of degradation in Indian style cooking [136]. Moreover, the estimated phytate content in the Wfood2 food composition tables has been between 353-845 mg and 20-617 mg per 100 g for the local staple grains and legumes, respectively. The phytate values in these food composition tables are comparatively lower than in the tables from other sources [137]. This indicates that our estimated phytate contribution could have been under- rather than overestimated.

5.6. Interpretation of nutritive and plasma zinc values

Due to the lack of national food composition table values for Nepal, we have used Indian food tables from the wfood2 computer software package [119]. The nutritive value may differ according to the soil [44], storage and processing of foods during cooking and other factors. Thus, the values, which we have obtained from Indian tables, may be somewhat different from those of the nutritive values of local foods. Similarly, for the interpretation of dietary adequacy indicators like EAR cut-offs, we have chosen international values set by the National Academy of Sciences, US [113], which may also over- or underestimate our intake results. The cut-off values set by the IZiNCG for the estimation of zinc deficiency was based on a healthy US population without infection on the day of blood sampling [131]. We have enrolled healthy women for the estimation of zinc deficiency but our enrolled children had acute respiratory illnesses which may have influenced their plasma zinc concentration [72]. Unfortunately, there are no standardized cut-off values indicating zinc deficiency by measuring plasma zinc during illness.

5.7. Zinc administration for the treatment of community acquired pneumonia

Although recent years have seen substantial reductions in child mortality and morbidity [138], one in every fifth childhood death is still caused by pneumonia [5, 139]. Most of these deaths are caused by bacterial pneumonias, which probably constitute at least 50% of hospitalized cases of pneumonia in children [78]. Reduction of the number of childhood pneumonia deaths is an important prerequisite for the achievement of the Millennium Development Goal-4 [140]. Improvement in zinc nutrition is considered as one of the effective ways to prevent childhood pneumonia together with increasing immunization coverage, exclusive breastfeeding prevalence and improving the children's general nutritional status [141]. We enrolled 2,628 episodes of community acquired pneumonia in 2-35 months old children and did not find any beneficial or harmful effect of oral zinc in terms of severity or duration of illness.

To our knowledge, this is the first study of the efficacy of zinc as adjuvant therapy in primarily non-severe community acquired pneumonia. We used the WHO/IMCI definition for the diagnosis of pneumonia, which is based on fast breathing. In this definition, the diagnostic specificity has been compromised in order to ensure high sensitivity [57, 78]. Apart from bacterial pneumonia, several other illnesses such as malaria, enteric fever, reactive airway disease, viral bronchiolitis, anemia and other febrile illnesses may also cause fast breathing [142-144]. Earlier, a positive effect of zinc was observed primarily when the etiology was of presumed bacterial origin [73, 80], though this notion was challenged by the results from a trial undertaken in India [83]. Thus, in a randomized double-blind trial in young children with pneumonia in Vellore, there were no overall effects of zinc as adjunct therapy. Having CRP

concentration > 40 mg/L, which indicate a bacterial etiology, was associated with a negative effect of zinc.

The mean baseline plasma zinc concentration in our study was 8.9 µmol/L (SD 2.6) which is lower than in the children included in earlier studies on pneumonia, where it ranged from 9.9 -11.0 µmol/L [145]. When using zinc for the treatment of acute diarrhea, low baseline zinc status was identified as one of the subgroups where zinc was particularly efficacious. By using the cut-off of IZiNCG (<9.9 µmol/L), 70% of our enrolled children were zinc deficient. However, we could not observe beneficial effect of zinc supplementation even in this sub-group of children with low plasma zinc.

We changed to second line antibiotics within 72 hours in 21% and 20% of the children receiving zinc or placebo, respectively. The standard WHO case management of pneumonia recommends a change to second line antibiotic after 48 hours [146], if there is no response by the first line antibiotic. Had we used this guideline, more than 50% of children would have required antibiotic changes, again equally distributed between the intervention arms (data not shown).

Resistance to antibiotics is a growing problem globally, particularly in developing countries [147]. In Nepal and in other countries more and more children with presumably viral cough and cold or diarrhea receive treatment with antibiotics [71, 148-150]. One of the concerns using the WHO/IMCI recommendations for treatment of non-severe pneumonia is that it increases the unnecessary use of antibiotics. The results from our study provided information that a substantial proportion of antibiotic prescription could be averted by prolonging duration of antibiotic change up to 72

hours without causing any further risk for children in our research setting. Recently, based on their follow up study among 876 children in Pakistan, Hazir *et al*. also suggested not to conclude with treatment failure if a child does not deteriorate from non-severe pneumonia even on day 3 [151].

The finding of vomiting/regurgitation in zinc recipients was consistent with our previous report from the same setting [63] and elsewhere [152]. Most of these vomiting/regurgitation episodes occurred on the first day of enrollment. The exact mechanism behind regurgitation and vomiting after zinc administration is not known but it is one of its most common side effects [153]. Vomiting during an illness such as pneumonia may lead to worsening of the clinical picture because of aspiration, particularly among young infants. Fortunately, in our trial, the zinc induced vomiting was not higher among the young children compared to the older study participants.

5.8. Zinc supplementation and delayed effects

Preventive zinc supplementation trials in developing countries have shown beneficial effects in terms of reduced incidence of common childhood illnesses such as pneumonia and diarrhea, where zinc deficiency is presumed to be high. In a pooled analysis published in 1999, 3 small short-course zinc supplementation trials from selected groups of children were included and the effect size on prevention of diarrheal and respiratory illness was comparable with that found with continuous preventive trials [54]. A subsequent study by Rahman *et al.* in Bangladesh [86, 154], found a reduction on diarrheal incidence but increased respiratory illness risk and no effect on growth for 6 months of follow up. Two additional zinc studies from Bangladesh by Brooks *et al.* [9] and Baqui *et al.* [155] reported substantial benefits in terms of morbidity and mortality reduction when zinc were given weekly for healthy

kids or intermittently during each episode of diarrhea. However, the results from trials measuring the effects of short-courses of zinc supplementation are not consistent. The children in our trial who received zinc during pneumonia did not have more or less infections over the subsequent six months compared to children that received placebo (Paper IV). Our findings of no effect in terms of reduction of incidence of diarrheal and respiratory illnesses is in line with the results from our own previous study based on one month of follow up [63] and a study by Fisher-Walker *et al*. [83], undertaken in 3 countries among children < 6 months of age with acute diarrhea.

In the present study with high precision, we have shown that zinc administration had no effect on the time until the first subsequent episode of non-severe pneumonia, severe pneumonia, diarrhea or dysentery. To record illnesses during the follow up period, we used different approaches. The field hospital is located within walking distance for more than 90% of the enrolled children and is one of the main established pediatric service centers in this area. Additionally, we provided free treatment facilities for enrolled children. So, we assume that we recorded most of the major illnesses from enrolled kids, which also confirmed by similarities on record between physicians and mother's recall.

Unlike vitamin A and iron, zinc is not stored in our body and its turnover is rapid, and zinc loss is augmented by frequent infections that are common among children in low income countries [156]. This may explain why beneficial effects were observed in continuous [55] or intermittent supplementation trials where zinc was given weekly [9] or in each episode of diarrhea [85] but not when zinc was given at one point in time [157, 158]. The plasma concentration in our study increased only until the administration period was over and not on 45 or 90 days after enrollment. So, it may

not be surprising that we could not see any beneficial effect on morbidity during the 3 and 6 months follow up period.

6. Main conclusions and recommendations

1. In a cross-sectional study among non-pregnant women in Bhaktapur, Nepal, the prevalence of anemia, iron deficiency and iron deficiency anemia were 12%, 20% and 6%, respectively. Most of the iron containing foods was non-heme sources like rice, green vegetables and wheat whereas heme iron contributed only 5% of the total iron intake. Age, parity and use of Depo-Provera contraceptive injection were positively associated with hemoglobin concentration.

2. The prevalence of iron deficiency and anemia in our study was substantially lower than the national estimates, which indicates that the prevalence may differ according to geographical location, dietary pattern and ethnic background. Local adaptation of the national guidelines for iron deficiency prevention programs may accordingly be needed. Carpet factory women, who represent women from other parts of the country, had a higher prevalence of iron deficiency and anemia than the local residents.

3. We also documented that zinc deficiency was very common in women of reproductive age, affecting 78%-90% depending upon the cut-offs used. The intake of zinc was not associated with plasma zinc concentration, while phytate intake was. The median intake of phytate was > 2 gram per day and

the mean P:Z molar ratio was 26:1, indicating profound inhibition of zinc absorption in many of our study participants.

4. The prevalence of zinc deficiency in women of reproductive age was substantially higher than the prevalence of iron deficiency in this community, and possible options for improvement of these nutrients include consumption of micronutrient enriched foods, dietary fortification and supplementation. The P: Z molar ratio and the intake of phytate can be reduced by milling, germination, fermentation and soaking of local foods before preparation and consumption.

5. Future studies should address possible health consequences of zinc deficiency, including in babies born to women with this deficiency.

6. Zinc administration as an adjuvant to standard antibiotic therapy among young Nepalese children neither reduced the treatment failure risk nor the duration of the pneumonia episode. Our study is not an argument for expanding oral zinc for management of acute lower respiratory tract infections in children.

7. Short-course zinc administration during community acquired childhood pneumonia increased plasma zinc concentration only until the last day of zinc

administration. To maintain proper zinc stores, intermittent or continuous supplementation may be necessary. The short-course zinc administration did not reduce the risk of respiratory or diarrheal illness during the 6 months of follow up. In future studies, priority should be given to identify subgroups of children who might benefit from short-course of zinc administration.

References:

1. Black, R.E., et al., *Maternal and child undernutrition: global and regional exposures and health consequences.* Lancet, 2008. **371**(9608): p. 243-60.
2. Victora, C.G., et al., *Maternal and child undernutrition: consequences for adult health and human capital.* Lancet, 2008. **371**(9609): p. 340-57.
3. Pelletier, D.L., E.A. Frongillo, Jr., and J.P. Habicht, *Epidemiologic evidence for a potentiating effect of malnutrition on child mortality.* Am J Public Health, 1993. **83**(8): p. 1130-3.
4. Caulfield, L.E., et al., *Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles.* Am J Clin Nutr, 2004. **80**(1): p. 193-8.
5. Bryce, J., et al., *WHO estimates of the causes of death in children.* Lancet, 2005. **365**(9465): p. 1147-52.
6. West, K.P., Jr., et al., *Double blind, cluster randomised trial of low dose supplementation with vitamin A or beta carotene on mortality related to pregnancy in Nepal. The NNIPS-2 Study Group.* Bmj, 1999. **318**(7183): p. 570-5.
7. Sazawal, S., et al., *Zinc supplementation in infants born small for gestational age reduces mortality: a prospective, randomized, controlled trial.* Pediatrics, 2001. **108**(6): p. 1280-6.

8. Tielsch, J.M., et al., *Effect of daily zinc supplementation on child mortality in southern Nepal: a community-based, cluster randomised, placebo-controlled trial.* Lancet, 2007. **370**(9594): p. 1230-9.

9. Brooks, W.A., et al., *Effect of weekly zinc supplements on incidence of pneumonia and diarrhoea in children younger than 2 years in an urban, low-income population in Bangladesh: randomised controlled trial.* Lancet, 2005. **366**(9490): p. 999-1004.

10. Bhandari, N., et al., *Adding zinc to supplemental iron and folic acid does not affect mortality and severe morbidity in young children.* J Nutr, 2007. **137**(1): p. 112-7.

11. Taneja, S., et al., *Effect of zinc supplementation on morbidity and growth in hospital-born, low-birth-weight infants.* The American journal of clinical nutrition, 2009. **90**(2): p. 385-91.

12. Sazawal, S., et al., *Effect of zinc supplementation on mortality in children aged 1-48 months: a community-based randomised placebo-controlled trial.* Lancet, 2007. **369**(9565): p. 927-34.

13. UNICEF, *The State of the world's children. United Nations Children Fund (UNICEF)*, 1998.

14. MOHP, *Nepal Demographic Health Survey 2006. Ministry of Health and Population (MOHP). New Era and Macro International Inc. Kathmandu.* 2007.

15. Fall, C.H., et al., *Micronutrients and fetal growth.* J Nutr, 2003. **133**(5 Suppl 2): p. 1747S-1756S.

16. Kramer, M.S., *Determinants of low birth weight: methodological assessment and meta-analysis.* Bull World Health Organ, 1987. **65**(5): p. 663-737.

17. Shrimpton, R., et al., *Worldwide timing of growth faltering: implications for nutritional interventions.* Pediatrics, 2001. **107**(5): p. E75.

18. Khambalia, A., D.L. O'Connor, and S. Zlotkin, *Periconceptional iron and folate status is inadequate among married, nulliparous women in rural Bangladesh.* J Nutr, 2009. **139**(6): p. 1179-84.

19. Jiang, T., et al., *Micronutrient deficiencies in early pregnancy are common, concurrent, and vary by season among rural Nepali pregnant women.* J Nutr, 2005. **135**(5): p. 1106-12.

20. Ronnenberg, A.G., et al., *Preconception hemoglobin and ferritin concentrations are associated with pregnancy outcome in a prospective cohort of Chinese women.* J Nutr, 2004. **134**(10): p. 2586-91.

21. Stoltzfus, R.J., *Iron deficiency: global prevalence and consequences.* Food Nutr Bull, 2003. **24**(4 Suppl): p. S99-103.

22. Dreyfuss, M.L., et al., *Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal.* J Nutr, 2000. **130**(10): p. 2527-36.

23. Bondevik, G.T., et al., *The prevalence of anemia in pregnant Nepali women--a study in Kathmandu.* Acta Obstet Gynecol Scand, 2000. **79**(5): p. 341-9.

24. Banhidy, F., et al., *Iron deficiency anemia: pregnancy outcomes with or without iron supplementation.* Nutrition, 2011. **27**(1): p. 65-72.

25. Singla, P.N., et al., *Fetal growth in maternal anaemia.* J Trop Pediatr, 1997. **43**(2): p. 89-92.

26. Murray-Kolb, L.E. and J.L. Beard, *Iron deficiency and child and maternal health.* Am J Clin Nutr, 2009. **89**(3): p. 946S-950S.

27. Stoltzfus, R., *Defining iron-deficiency anemia in public health terms: a time for reflection.* J Nutr, 2001. **131**(2S-2): p. 565S-567S.

28. WHO, *Iron and folate supplementation. Integrated management of pregnancy and childbirth (IMPAC)*, 2006, WHO: Geneva.

29. WHO, *Iron Deficiency Anemia. Assessment, Prevention and Control. A guide for programme manager*, 2001. p. 114.

30. Gera, T. and H.P. Sachdev, *Effect of iron supplementation on incidence of infectious illness in children: systematic review.* Bmj, 2002. **325**(7373): p. 1142.

31. Lind, T., et al., *A community-based randomized controlled trial of iron and zinc supplementation in Indonesian infants: interactions between iron and zinc.* Am J Clin Nutr, 2003. **77**(4): p. 883-90.

32. Tielsch, J.M., et al., *Effect of routine prophylactic supplementation with iron and folic acid on preschool child mortality in southern Nepal: community-based, cluster-randomised, placebo-controlled trial.* Lancet, 2006. **367**(9505): p. 144-52.

33. Sazawal, S., et al., *Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomised, placebo-controlled trial.* Lancet, 2006. **367**(9505): p. 133-43.

34. WHO, *Iron supplements of young children in regions where malaria transmission is intense and infectious disease is highly prevalent-WHO statement. (http://www.who.int/child_adolescent_health/documents/pdfs/who_statement_iron.pdf)* 2006, World Health Organization: Geneva, Switzerland.

35. De Benoist, B., et al., *Zinc and iron supplementation trials in Nepal and Tanzania.* Lancet, 2006. **367,816**.

36. Iannotti, L.L., et al., *Iron supplementation in early childhood: health benefits and risks.* Am J Clin Nutr, 2006. **84**(6): p. 1261-76.

37. Fraker, P.J., et al., *The dynamic link between the integrity of the immune system and zinc status.* J Nutr, 2000. **130**(5S Suppl): p. 1399S-406S.

38. Shankar, A.H. and A.S. Prasad, *Zinc and immune function: the biological basis of altered resistance to infection.* Am J Clin Nutr, 1998. **68**(2 Suppl): p. 447S-463S.

39. Prasad, A.S., *Zinc: role in immunity, oxidative stress and chronic inflammation.* Curr Opin Clin Nutr Metab Care, 2009. **12**(6): p. 646-52.

40. Hotz, C. and K.H. Brown, *Assessment of the risk of zinc deficiency in populations and options for its control. International Zinc Nutrition*

Consultative Group (IZiNCG). Technical document. Food Nutr Bull, 2004. **25**(S91): p. S91-S204.

41. Walsh, C.T., et al., *Zinc: health effects and research priorities for the 1990s.* Environ Health Perspect, 1994. **102 Suppl 2**: p. 5-46.
42. Fischer Walker, C.L., M. Ezzati, and R.E. Black, *Global and regional child mortality and burden of disease attributable to zinc deficiency.* Eur J Clin Nutr, 2009. **63**(5): p. 591-7.
43. Ma, G., et al., *Phytate intake and molar ratios of phytate to zinc, iron and calcium in the diets of people in China.* Eur J Clin Nutr, 2006.
44. Alloway, B.J., *Zinc in Soil and Crop Nutrition*. Second ed2008: International Zinc Association and International Fertilizer Industry Association. Brussels, Belgium and Peris, France.
45. IZiNCG, *Executive summary. Recommendations for indicators of population zinc status. Report of WHO/ UNICEF/IAEA/IZiNCG Interagency Meeting on Zinc Status Indicators.* Food Nutr Bull, 2007. **28**: p. S399-S400.
46. King, J.C., *Determinants of maternal zinc status during pregnancy.* Am J Clin Nutr, 2000. **71**(5 Suppl): p. 1334S-43S.
47. Rwebembera, A.A., et al., *Relationship between infant birth weight </=2000 g and maternal zinc levels at Muhimbili National Hospital, Dar Es Salaam, Tanzania.* J Trop Pediatr, 2006. **52**(2): p. 118-25.

48. Osendarp, S.J., C.E. West, and R.E. Black, *The need for maternal zinc supplementation in developing countries: an unresolved issue.* J Nutr, 2003. **133**(3): p. 817S-827S.

49. Prasad, A.S., *Zinc deficiency in women, infants and children.* J Am Coll Nutr, 1996. **15**(2): p. 113-20.

50. Caulfield, L.E., et al., *Potential contribution of maternal zinc supplementation during pregnancy to maternal and child survival.* Am J Clin Nutr, 1998. **68**: p. 499S-508S.

51. Seshadri, S., *Prevalence of micronutrient deficiency particularly of iron, zinc and folic acid in pregnant women in South East Asia.* Br J Nutr, 2001. **85 Suppl 2**: p. S87-92.

52. Mahomed, K., Z. Bhutta, and P. Middleton, *Zinc supplementation for improving pregnancy and infant outcome.* Cochrane Database Syst Rev, 2007(2): p. CD000230.

53. Ronsmans, C., et al., *Multiple micronutrient supplementation during pregnancy in low-income countries: a meta-analysis of effects on stillbirths and on early and late neonatal mortality.* Food and nutrition bulletin, 2009. **30**(4 Suppl): p. S547-55.

54. Bhutta, Z.A., et al., *Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled analysis of randomized controlled trials. Zinc Investigators' Collaborative Group.* J Pediatr, 1999. **135**(6): p. 689-97.

55. Aggarwal, R., J. Sentz, and M.A. Miller, *Role of zinc administration in prevention of childhood diarrhea and respiratory illnesses: a meta-analysis.* Pediatrics, 2007. **119**(6): p. 1120-30.

56. Roth, D.E., S.A. Richard, and R.E. Black, *Zinc supplementation for the prevention of acute lower respiratory infection in children in developing countries: meta-analysis and meta-regression of randomized trials.* Int J Epidemiol, 2010. **39**(3): p. 795-808.

57. Bhandari, N., et al., *Effect of routine zinc supplementation on pneumonia in children aged 6 months to 3 years: randomised controlled trial in an urban slum.* Bmj, 2002. **324**(7350): p. 1358.

58. Bhutta, Z.A., et al., *What works? Interventions for maternal and child undernutrition and survival.* Lancet, 2008. **371**(9610): p. 417-40.

59. Brown, K.H., et al., *Effect of supplemental zinc on the growth and serum zinc concentrations of prepubertal children: a meta-analysis of randomized controlled trials.* Am J Clin Nutr, 2002. **75**(6): p. 1062-71.

60. Umeta, M., et al., *Zinc supplementation and stunted infants in Ethiopia: a randomised controlled trial.* Lancet, 2000. **355**(9220): p. 2021-6.

61. Ramakrishnan, U., P. Nguyen, and R. Martorell, *Effects of micronutrients on growth of children under 5 y of age: meta-analyses of single and multiple nutrient interventions.* Am J Clin Nutr, 2009. **89**(1): p. 191-203.

62. Taneja, S., et al., *Zinc supplementation for four months does not affect growth in young north Indian children.* The Journal of nutrition, 2010. **140**(3): p. 630-4.

63. Strand, T.A., et al., *Effectiveness and efficacy of zinc for the treatment of acute diarrhea in young children.* Pediatrics, 2002. **109**(5): p. 898-903.

64. Bhandari, N., et al., *Substantial reduction in severe diarrheal morbidity by daily zinc supplementation in young north Indian children.* Pediatrics, 2002. **109**(6): p. e86.

65. Bhutta, Z.A., et al., *Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials.* Am J Clin Nutr, 2000. **72**(6): p. 1516-22.

66. WHO, *World Health Organization (WHO) and United Nations Children's Fund (UNICEF). Joint statement on the Clinical Management of Acute Diarrhea* 2004, WHO and UNICEF: Geneva and New York.

67. Lukacik, M., R.L. Thomas, and J.V. Aranda, *A meta-analysis of the effects of oral zinc in the treatment of acute and persistent diarrhea.* Pediatrics, 2008. **121**(2): p. 326-36.

68. Baqui, A.H., et al., *Zinc therapy for diarrhoea increased the use of oral rehydration therapy and reduced the use of antibiotics in Bangladeshi children.* J Health Popul Nutr, 2004. **22**(4): p. 440-2.

69. Awasthi, S., *Zinc supplementation in acute diarrhea is acceptable, does not interfere with oral rehydration, and reduces the use of other medications: a*

randomized trial in five countries. J Pediatr Gastroenterol Nutr, 2006. **42**(3): p. 300-5.

70. Bhandari, N., et al., *Effectiveness of zinc supplementation plus oral rehydration salts compared with oral rehydration salts alone as a treatment for acute diarrhea in a primary care setting: a cluster randomized trial.* Pediatrics, 2008. **121**(5): p. e1279-85.

71. Reynolds, L. and M. McKee, *Factors influencing antibiotic prescribing in China: an exploratory analysis.* Health Policy, 2009. **90**(1): p. 32-6.

72. Strand, T.A., et al., *Predictors of plasma zinc concentrations in children with acute diarrhea.* Am J Clin Nutr, 2004. **79**(3): p. 451-6.

73. Mahalanabis, D., et al., *Randomized, double-blind, placebo-controlled clinical trial of the efficacy of treatment with zinc or vitamin A in infants and young children with severe acute lower respiratory infection.* Am J Clin Nutr, 2004. **79**(3): p. 430-6.

74. Rudan, I., et al., *Epidemiology and etiology of childhood pneumonia.* Bull World Health Organ, 2008. **86**(5): p. 408-16.

75. Grijalva, C.G., et al., *Decline in pneumonia admissions after routine childhood immunisation with pneumococcal conjugate vaccine in the USA: a time-series analysis.* Lancet, 2007. **369**(9568): p. 1179-86.

76. Nelson, J.C., et al., *Impact of the introduction of pneumococcal conjugate vaccine on rates of community acquired pneumonia in children and adults.* Vaccine, 2008. **26**(38): p. 4947-54.

77. Baqui, A.H., et al., *Effectiveness of Haemophilus influenzae type B conjugate vaccine on prevention of pneumonia and meningitis in Bangladeshi children: a case-control study.* Pediatr Infect Dis J, 2007. **26**(7): p. 565-71.

78. WHO. *World Health Organization (1991). Programme for the control of acute respiratory infections. Technical bases for the WHO recommendations on the management of pneumonia in children at first-level health facilities. Version current 9 August 1991* 15.06.10]; Available from: http://whqlibdoc.who.int/hq/1991/WHO_ARI_91.20.pdf.

79. Polack, F.P. and R.A. Karron, *The future of respiratory syncytial virus vaccine development.* The Pediatric Infectious Disease Journal, 2004. **23**(1): p. S65-S73.

80. Brooks, W.A., et al., *Zinc for severe pneumonia in very young children: double-blind placebo-controlled trial.* Lancet, 2004. **363**(9422): p. 1683-8.

81. Mahalanabis, D., et al., *Zinc supplementation as adjunct therapy in children with measles accompanied by pneumonia: a double-blind, randomized controlled trial.* Am J Clin Nutr, 2002. **76**(3): p. 604-7.

82. Bose, A., et al., *Efficacy of zinc in the treatment of severe pneumonia in hospitalized children <2 y old.* Am J Clin Nutr, 2006. **83**(5): p. 1089-96; quiz 1207.

83. Coles, C.L., et al., *Infectious etiology modifies the treatment effect of zinc in severe pneumonia.* Am J Clin Nutr, 2007. **86**(2): p. 397-403.

84. Allen, L.H. and S.R. Gillespie, *What works? A review of the efficacy and effectiveness of nutrition interventions. Geneva: United Nations Administrative Committee on Coordination/Sub-Committee on Nutrition (ACC/SCN) and Manila: Asian Development Bank*, 2001.

85. Baqui, A.H., et al., *Effect of zinc supplementation started during diarrhoea on morbidity and mortality in Bangladeshi children: community randomised trial.* Bmj, 2002. **325**(7372): p. 1059.

86. Rahman, M.M., et al., *Simultaneous zinc and vitamin A supplementation in Bangladeshi children: randomised double blind controlled trial.* Bmj, 2001. **323**(7308): p. 314-8.

87. Walker, C.L. and R.E. Black, *Zinc for the treatment of diarrhoea: effect on diarrhoea morbidity, mortality and incidence of future episodes.* International journal of epidemiology, 2010. **39 Suppl 1**: p. i63-9.

88. Jones, G., et al., *How many child deaths can we prevent this year?* Lancet, 2003. **362**(9377): p. 65-71.

89. de Benoist, B., et al., *Conclusions of the Joint WHO/UNICEF/IAEA/IZiNCG Interagency Meeting on Zinc Status Indicators.* Food Nutr Bull, 2007. **28**(3 Suppl): p. S480-4.

90. Hotz, C., et al., *Assessment of the trace element status of individuals and populations: the example of zinc and copper.* J Nutr, 2003. **133**(5 Suppl 1): p. 1563S-8S.

91. Fischer Walker, C.L. and R.E. Black, *Functional indicators for assessing zinc deficiency.* Food Nutr Bull, 2007. **28**(3 Suppl): p. S454-79.

92. Gibson, R.S. and E.L. Ferguson, *An Interactive 24-hour Recall for Assessing the adequacy of Iron and Zinc Intakes in Developing Countries*1999, Washington, DC: International Life Sciences Institute.

93. Mohamed, A.A., et al., *Zinc, parity, infection, and severe anemia among pregnant women in kassla, eastern Sudan.* Biological trace element research, 2011. **140**(3): p. 284-90.

94. Zimmermann, M.B., et al., *Vitamin A supplementation in children with poor vitamin A and iron status increases erythropoietin and hemoglobin concentrations without changing total body iron.* Am J Clin Nutr, 2006. **84**(3): p. 580-6.

95. Bondevik, G.T., et al., *Homocysteine and methylmalonic acid levels in pregnant Nepali women. Should cobalamin supplementation be considered?* Eur J Clin Nutr, 2001. **55**(10): p. 856-64.

96. Hegazy, A.A., et al., *Relation between anemia and blood levels of lead, copper, zinc and iron among children.* BMC research notes, 2010. **3**: p. 133.

97. Eskeland, B., et al., *Influence of mild infections on iron status parameters in women of reproductive age.* Scand J Prim Health Care, 2002. **20**(1): p. 50-6.

98. Skikne, B.S., C.H. Flowers, and J.D. Cook, *Serum transferrin receptor: a quantitative measure of tissue iron deficiency.* Blood, 1990. **75**(9): p. 1870-6.

99. Punnonen, K., K. Irjala, and A. Rajamaki, *Serum transferrin receptor and its ratio to serum ferritin in the diagnosis of iron deficiency.* Blood, 1997. **89**(3): p. 1052-7.

100. Olivares, M., et al., *Usefulness of serum transferrin receptor and serum ferritin in diagnosis of iron deficiency in infancy.* Am J Clin Nutr, 2000. **72**(5): p. 1191-5.

101. Thomas, C. and L. Thomas, *Biochemical markers and hematologic indices in the diagnosis of functional iron deficiency.* Clin Chem, 2002. **48**(7): p. 1066-76.

102. Dijkhuizen, M.A., et al., *Concurrent micronutrient deficiencies in lactating mothers and their infants in Indonesia.* Am J Clin Nutr, 2001. **73**(4): p. 786-91.

103. Wieringa, F.T., et al., *Combined iron and zinc supplementation in infants improved iron and zinc status, but interactions reduced efficacy in a multicountry trial in southeast Asia.* J Nutr, 2007. **137**(2): p. 466-71.

104. Fischer Walker, C., et al., *Interactive effects of iron and zinc on biochemical and functional outcomes in supplementation trials.* Am J Clin Nutr, 2005. **82**(1): p. 5-12.

105. Crofton, R.W., et al., *Inorganic zinc and the intestinal absorption of ferrous iron.* Am J Clin Nutr, 1989. **50**(1): p. 141-4.

106. Fairweather-Tait, S.J., S.G. Wharf, and T.E. Fox, *Zinc absorption in infants fed iron-fortified weaning food.* Am J Clin Nutr, 1995. **62**(4): p. 785-9.

107. Mathisen, M., et al., *RNA viruses in community-acquired childhood pneumonia in semi-urban Nepal; a cross-sectional study.* BMC Med, 2009. **7**: p. 35.

108. Mathisen, M., et al., *Clinical presentation and severity of viral community-acquired pneumonia in young Nepalese children.* The Pediatric Infectious Disease Journal, 2010. **29**(1): p. e1-6.

109. Mathisen, M., et al., *Respiratory viruses in nepalese children with and without pneumonia: a case-control study.* The Pediatric Infectious Disease Journal, 2010. **29**(8): p. 731-5.

110. CBS, *District Profiles*, 2007, The Planning, Coordination and Standardization Section. Government of Nepal, Central Bureau of Statistics (CBS).

111. CBS, *Four - Monthly Statisitical Bulletin. Central Bureau of Statistics (CBS), National Planning Comission, Nepal Government*, 2008. p. 27.

112. DPHO, *Half Yearly report on EPI coverage in Bhaktapur district*, 2008, District Public Health Office (DPHO), Bhaktapur.

113. DRI, *Dietary References Intakes (DRI):Application in Dietary Planning. Subcommittee on Interpretation and Uses of Dietary Reference Intakes and the Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. National Academy Press.*, 2003, Institute of Medicine of the national academes: Washington, D.C.

114. IMCI, *Integrated Management of Childhood Illness (IMCI) - Handbook*, 2003, Department of Child and Adolescent Health and Development. World Health Organization, Geneva, Switzerland.

115. CDC, *Centers for Disease Control (CDC). CDC Criteria for anemiain children and child bearing aged women.* CDC MMWR, 1989. **38**: p. 400-4.

116. Hallberg, L., et al., *Screening for iron deficiency: an analysis based on bone-marrow examinations and serum ferritin determinations in a population sample of women.* Br J Haematol, 1993. **85**(4): p. 787-98.

117. IZiNCG, *International Zinc Nutrition Consultative Group (IZiNCG). Quantifying the risk of zinc deficiency: Recommended indicators. IZiNCG Techinical brief No.1 Davis,CA*, 2007.

118. IZiNCG, *Assessing population zinc status with serum zinc concentration. International Zinc Nutrition Consultative Group (IZiNCG). Technical Brief. No 2. 2007*, K. Brown, Editor 2007.

119. Wfood2, *World food 2 computer software pockage. Version 1.0.The Regents of the University of California.* 1996.

120. NMSS, *Nepal Micronutrient Status Survey (NMSS),1998. Ministry of Health, Child Health Division, HMG/N, New Era, Micronutrient Initiative, UNICEF Nepal and WHO*, 2001: Kathmandu.

121. Guyatt, G.H., et al., *Laboratory diagnosis of iron-deficiency anemia: an overview.* Journal of general internal medicine, 1992. **7**(2): p. 145-53.

122. Gibson, R.S., et al., *Zinc, gravida, infection, and iron, but not vitamin B-12 or folate status, predict hemoglobin during pregnancy in Southern Ethiopia.* J Nutr, 2008. **138**(3): p. 581-6.

123. Dreyfuss, M.L., et al., *Hookworms, malaria and vitamin A deficiency contribute to anemia and iron deficiency among pregnant women in the plains of Nepal.* The Journal of nutrition, 2000. **130**(10): p. 2527-36.

124. Chandyo, R.K., *Cobalamin and folate status among women of reproductive age in Bhaktapur, Nepal*, in *Centre for International Health*2002, University of Bergen: Bergen. p. 83.

125. IZiNCG, *Determining the risk of zinc deficiency: Assessment of dietary zinc intake. Technical Brief. No.03. 2007*, R. Gibson, Editor 2007.

126. Pathak, P., et al., *Magnitude of zinc deficiency among nulliparous nonpregnant women in a rural community of Haryana State, India.* Food Nutr Bull, 2003. **24**(4): p. 368-71.

127. Gibson, R.S. and J.M. Huddle, *Suboptimal zinc status in pregnant Malawian women: its association with low intakes of poorly available zinc, frequent reproductive cycling, and malaria.* Am J Clin Nutr, 1998. **67**(4): p. 702-9.

128. Abebe, Y., et al., *Inadequate intakes of dietary zinc among pregnant women from subsistence households in Sidama, Southern Ethiopia.* Public Health Nutr, 2008. **11**(4): p. 379-86.

129. de Jong, N., A.B. Romano, and R.S. Gibson, *Zinc and iron status during pregnancy of Filipino women.* Asia Pac J Clin Nutr, 2002. **11**(3): p. 186-93.

130. IOM, *Institute of Medicine. Dietary Reference Intakes for Vitamin A, Vitamin K, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc. Food and Nutrition Board, Washington, DC: National Academy Press*, 2001.

131. Hotz, C., J.M. Peerson, and K.H. Brown, *Suggested lower cutoffs of serum zinc concentrations for assessing zinc status: reanalysis of the second National Health and Nutrition Examination Survey data (1976-1980).* Am J Clin Nutr, 2003. **78**(4): p. 756-64.

132. Hambidge, K.M., et al., *Post-prandial and daily changes in plasma zinc.* J Trace Elem Electrolytes Health Dis, 1989. **3**(1): p. 55-7.

133. Markowitz, M.E., J.F. Rosen, and M. Mizruchi, *Circadian variations in serum zinc (Zn) concentrations: correlation with blood ionized calcium, serum total calcium and phosphate in humans.* Am J Clin Nutr, 1985. **41**(4): p. 689-96.

134. Wallock, L.M., et al., *Meal-induced changes in plasma, erythrocyte, and urinary zinc concentrations in adult women.* Am J Clin Nutr, 1993. **58**(5): p. 695-701.

135. Harland, B.F. and D. Oberleas, *Phytate in foods.* World Rev Nutr Diet, 1987. **52**: p. 235-59.

136. Agte, V.V., K.V. Tarwadi, and S.A. Chiplonkar, *Phytate degradation during traditional cooking: significance of the phytic acid profile in cereal based vegetarian meals.* J. Food Compost Anal, 1999. **12**: p. 161-67.

137. Andersen, P. and R.K. Chandyo, *Nutritional problems among women of reproductive age in rural populations in Hill areas in India and Nepal and how they can be reduced by increased intake of Ricebean*, 2010.

138. Ahmad, O.B., A.D. Lopez, and M. Inoue, *The decline in child mortality: a reappraisal.* Bull World Health Organ, 2000. **78**(10): p. 1175-91.

139. Williams, B.G., et al., *Estimates of world-wide distribution of child deaths from acute respiratory infections.* Lancet Infect Dis, 2002. **2**(1): p. 25-32.

140. Bryce, J., et al., *Countdown to 2015: tracking intervention coverage for child survival.* Lancet, 2006. **368**(9541): p. 1067-76.

141. UNICEF. *Pneumonia: The forgotton Killer of children.* 2006 15.06.2009]; Available from: http://www.unicef.org/publications/files/Pneumonia_The_Forgotten_Killer_of_Children.pdf.

142. Meremikwu, M.M., et al., *Hyperventilation in febrile Nigerian children without pneumonia: an evaluation of the influence of anaemia.* Cent Afr J Med, 1995. **41**(8): p. 248-52.

143. O'Dempsey, T.J., et al., *The effect of temperature reduction on respiratory rate in febrile illnesses.* Arch Dis Child, 1993. **68**(4): p. 492-5.

144. Hazir, T., et al., *Assessment and management of children aged 1-59 months presenting with wheeze, fast breathing, and/or lower chest indrawing; results of a multicentre descriptive study in Pakistan.* Arch Dis Child, 2004. **89**(11): p. 1049-54.

145. Natchu, U.C., M.R. Fataki, and W.W. Fawzi, *Zinc as an adjunct for childhood pneumonia - interpreting early results.* Nutr Rev, 2008. **66**(7): p. 398-405.

146. WHO, *World Health Organization. Integrated Management of Childhood Illness (IMCI) chart booklet. WHO/UNICEF, Geneva*, 2008.

147. Gill, C.J., et al., *Effect of presumptive co-trimoxazole prophylaxis on pneumococcal colonization rates, seroepidemiology and antibiotic resistance in Zambian infants: a longitudinal cohort study.* Bull World Health Organ, 2008. **86**(12): p. 929-38.

148. Shankar, R.P., et al., *Prescribing patterns of antibiotics and sensitivity patterns of common microorganisms in the Internal Medicine ward of a teaching hospital in Western Nepal: a prospective study.* Ann Clin Microbiol Antimicrob, 2003. **2**: p. 7.

149. Wachter, D.A., M.P. Joshi, and B. Rimal, *Antibiotic dispensing by drug retailers in Kathmandu, Nepal.* Trop Med Int Health, 1999. **4**(11): p. 782-8.

150. Harnden, A., et al., *Respiratory infections for which general practitioners consider prescribing an antibiotic: a prospective study.* Arch Dis Child, 2007. **92**(7): p. 594-7.

151. Hazir, T., et al., *Can WHO therapy failure criteria for non-severe pneumonia be improved in children aged 2-59 months?* Int J Tuberc Lung Dis, 2006. **10**(8): p. 924-31.

152. Larson, C.P., et al., *Initiation of zinc treatment for acute childhood diarrhoea and risk for vomiting or regurgitation: a randomized, double-blind, placebo-controlled trial.* J Health Popul Nutr, 2005. **23**(4): p. 311-9.

153. Khan, A.M., et al., *Introduction of routine zinc therapy for children with diarrhoea: evaluation of safety.* J Health Popul Nutr, 2007. **25**(2): p. 127-33.

154. Rahman, M.M., et al., *Short-term supplementation with zinc and vitamin A has no significant effect on the growth of undernourished Bangladeshi children.* Am J Clin Nutr, 2002. **75**(1): p. 87-91.

155. Baqui, A.H., et al., *Simultaneous weekly supplementation of iron and zinc is associated with lower morbidity due to diarrhea and acute lower respiratory infection in Bangladeshi infants.* J Nutr, 2003. **133**(12): p. 4150-7.

156. Brown, K.H., *Effect of infections on plasma zinc concentration and implications for zinc status assessment in low-income countries.* Am J Clin Nutr, 1998. **68**(2 Suppl): p. 425S-429S.

157. Valentiner-Branth, P., et al., *A randomized controlled trial of the effect of zinc as adjuvant therapy in children 2-35 mo of age with severe or nonsevere pneumonia in Bhaktapur, Nepal.* The American journal of clinical nutrition, 2010. **91**(6): p. 1667-74.

158. Walker, C.L., et al., *Zinc during and in convalescence from diarrhea has no demonstrable effect on subsequent morbidity and anthropometric status among infants <6 mo of age.* Am J Clin Nutr, 2007. **85**(3): p. 887-94.