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**Maternal micronutrient supplementation with zinc and  $\beta$ -carotene affects morbidity and immune function of infants during the first 6 months of life.**

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Running title: Prenatal zinc and  $\beta$ -carotene and morbidity in infants.

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**Abstract**

2 **Background/Objectives.** Micronutrient deficiencies are prevalent worldwide, and a  
3 major cause of infant death. Supplementation with multiple micronutrients during  
4 pregnancy might improve micronutrient status of the newborn, thereby reducing  
5 morbidity and death. Moreover, maternal supplementation might affect the  
6 newborn's immune development. Therefore, the present study investigated the effects  
7 of maternal zinc and  $\beta$ -carotene supplementation on the infant's morbidity and  
8 immune function during the first 6 mo of life.

9 **Subjects/Methods.** Mothers were supplemented during pregnancy with  $\beta$ -carotene  
10 and/or zinc, in addition to iron and folic acid, in a randomised, double-blind  
11 controlled trial. Newborn infants (n=136) were followed for six months.

12 **Results.** Infants born from mothers receiving zinc during pregnancy had significantly  
13 fewer episodes of diarrhoea than infants born from mothers not receiving zinc (0.2  
14 and 0.4, respectively) but more episodes of cough (1.3 and 0.9 respectively) during  
15 the first 6 mo. Maternal  $\beta$ -carotene supplementation had no effect on infants'  
16 morbidity. Cytokine production in the newborns was affected by maternal zinc and  $\beta$ -  
17 carotene supplementation, with zinc supplementation giving higher IL-6 production  
18 (16% higher), and  $\beta$ -carotene supplementation leading to lower IFN- $\gamma$  production  
19 (36% lower).

20 **Conclusions.** This study shows that maternal supplementation with zinc and  $\beta$ -  
21 carotene affected the newborn's immune development in specific ways, but only  
22 maternal zinc supplementation significantly affected morbidity in the infants.  
23 Addition of zinc to routine iron and folic acid supplements for pregnant women could  
24 be an effective way to reduce diarrheal disease during the first 6 mo of life albeit at  
25 the expense of more episodes of cough.

26

27 *Wordcount 248*

28

29 **KEYWORDS:** infant; vitamin A; deficiency; zinc; pregnancy; micronutrient; cytokine

30

## 2 INTRODUCTION

3 Micronutrient deficiency during infancy and childhood, especially deficiency of  
4 vitamin A and zinc, leads to an increased risk of morbidity and mortality from  
5 infectious diseases, and contributes to >1 million child deaths a year (Beaton *et al.*,  
6 1992; Sazawal *et al.*, 1998; Black *et al.*, 2008). Maternal nutritional status can be an  
7 important factor determining the micronutrient status of a breastfed infant (Dijkhuizen  
8 *et al.*, 2001; Dijkhuizen *et al.*, 2004; Baylin *et al.*, 2005). Supplementation with  
9 micronutrients during pregnancy has the advantage of addressing the needs of both  
10 mothers and infants simultaneously.

11

12 Vitamin A supplementation reduces mortality in children between 1 - 5 years  
13 by an estimated 23% (Beaton *et al.*, 1992). Several approaches to reduce vitamin A  
14 deficiency in children <1 year of age have been implemented, including intermittent  
15 high dose vitamin A supplementation of infants, and single high dose vitamin A  
16 supplementation of the mother directly post-partum (WHO, 1998), but these  
17 interventions have not been very successful in improving vitamin A status of infants  
18 (Ayah *et al.*, 2007). Moreover, effects of vitamin A supplementation in infants on  
19 morbidity and mortality have been disappointing (Malaba *et al.*, 2005). For example,  
20 a recent trial in Mexico reported a 27% increase in diarrheal disease episodes and a  
21 23% increase in fever episodes in infants receiving vitamin A supplementation every  
22 2 months, although the effect was dependent on housing conditions (Long *et al.*,  
23 2006).

24

25 Zinc supplementation in children has been associated with reductions in  
26 morbidity from infectious diseases, especially diarrheal and respiratory infections  
27 (Bhutta *et al.*, 2000), and zinc supplementation during pregnancy reduced the  
28 incidence and prevalence of diarrheal disease in low birthweight newborns (Osendarp  
29 *et al.*, 2001). Surprisingly, 2 large trials of iron and zinc supplementation in children  
30 failed to find a significant beneficial effect of zinc supplementation on mortality in

2 children under the age of 12 months (Sazawal *et al.*, 2007; Tielsch *et al.*, 2007).

3 Hence, whereas there appears to be beneficial effects of supplementation with vitamin  
4 A and zinc in children above 12 months of age, evidence is less clear for infants.

5

6 Both vitamin A and zinc are known to have immuno-modulating properties.  
7 Vitamin A deficiency favours the development of cellular (or Th1-cell) immune  
8 responses, whereas vitamin A supplementation appears to stimulate humoral (or Th2-  
9 cell) responses (Stephensen, 2001; Schuster *et al.*, 2008). Zinc deficiency leads to a  
10 general suppression of immune reactivity with lower production of cytokines of both  
11 Th-1 or Th-2 type being reported (Rink & Kirchner, 2000; Wieringa *et al.*, 2004), and  
12 supplementation with zinc restores decreased cytokine responses. The development of  
13 the human immune system begins at embryogenesis, and pre-natal exposure to  
14 immune-modulating agents can have long-lasting effects throughout childhood and  
15 into adult life as shown for e.g. organic toxins (Hertz-Picciotto *et al.*, 2008).

16

17 The present study investigated the effects of supplementation during  
18 pregnancy with zinc and/or  $\beta$ -carotene, in addition to iron and folic acid, on morbidity  
19 of the newborn infants during the first 6 mo of life and on immune responses 6 mo  
20 after birth. Furthermore, the 2x2 factorial design of the study allowed exploration of  
21 possible interactions between zinc and  $\beta$ -carotene on morbidity and immune function.

22

## 23 SUBJECTS AND METHODS

### 24 **Study design and location.**

25 The study was a follow-up study of a randomised, double-blind, controlled  
26 supplementation trial in 229 pregnant women with a gestational age <20 weeks, living  
27 in a rural area in West Java, Indonesia (Dijkhuizen *et al.*, 2004). All women were  
28 supplemented with iron and folic acid (30 mg of iron as ferrous fumarate and 0.4 mg  
29 of folic acid per day). In addition to iron and folic acid, one group of women received  
30  $\beta$ -carotene (4.5 mg per day), one group zinc (30 mg of zinc as sulphate per day), and

2 one group  $\beta$ -carotene plus zinc (4.5 mg and 30 mg per day, respectively).  
3 Supplements were given as capsules, and taken daily until delivery. Infants were born  
4 between November 1998 and July 1999. Infants and mothers were seen monthly until  
5 the infants were 6 mo old. Sample size calculations were primarily for micronutrient  
6 status outcomes, for which we had calculated a minimum sample size of 63 infants  
7 per group to detect a difference in retinol concentrations of 0.10  $\mu\text{mol/L}$ , with an SD  
8 of 0.20  $\mu\text{mol/L}$ . Moreover, an earlier study on cytokine production in infants  
9 receiving iron, zinc or  $\beta$ -carotene supplementation had shown differences of around  
10 11  $\mu\text{g/L}$  in IL-6 production (with an SD of 19  $\mu\text{g/L}$ ) and 340 ng/L of IFN- $\gamma$   
11 production (with an SD of 600 ng/L), requiring a sample size of 40 and 49 infants  
12 respectively. The 2x2 factorial design furthermore enables combining of groups in  
13 case there are no interactions between zinc and  $\beta$ -carotene supplementation. Finally a  
14 sample size of 50 children per supplement (zinc or  $\beta$ -carotene) would allow detection  
15 of a difference of >25% in the incidence of diarrhoea, assuming that 40% or less of  
16 the children in the control group experience at least 1 episode of diarrhoea.

17

### 18 **Subjects and procedures.**

19 The women had been informed at the beginning of supplementation and  
20 written informed consent was given. Exclusion criteria at enrolment were twin  
21 pregnancy and congenital abnormalities. A blood sample (5 mL) was collected at the  
22 end of the study of mother and infant for assessment of micronutrient status and  
23 immune responses. Morbidity was assessed monthly with a structured recall of signs  
24 and symptoms observed by the mother during the previous weeks, and recorded per  
25 week. A similar recall used earlier in the same study area was shown to give almost  
26 identical results as a daily morbidity record obtained by village health volunteers  
27 (unpublished data). The recall focused on symptoms of fever, cough, shortness of  
28 breath, diarrhoea (>3 watery stools per day) and vomiting, in accordance to local  
29 perceptions and using standard definitions and local terms.

30

## 2 **Methods.**

3 Non-fasting, morning blood samples were collected using a closed-tube  
4 vacuum system (Becton and Dickinson; Leiden, The Netherlands). Blood samples  
5 were immediately stored at 4°C and separated within 5 h. Plasma and serum samples  
6 were aliquoted and stored at -30°C until analysis.

7 Cytokine production was measured using an *ex-vivo* whole blood stimulation  
8 assay (Wieringa *et al.*, 2004) in 110 infants only, as blood collection was insufficient  
9 in 26 infants to perform the assay. Within 15 minutes, 200  $\mu$ l of whole blood, was  
10 diluted 1:1 with RPMI (RPMI 1640, Dutch Modification, Costar, Badhoevedorp, The  
11 Netherlands, with pyruvate, glutamin and gentamycin 1% w/v each), and incubated  
12 with either phytohaemagglutinin (PHA-P, Sigma, Zwijndrecht, The Netherlands) in a  
13 final concentration of 30 mg/L, lipopolysaccharide (LPS, *E. coli* serotype 055:b5;  
14 Sigma) in a final concentration of 10  $\mu$ g/L, or PBS as blank control, and then  
15 incubated at 37°C for 10 h. After incubation, the supernatant was collected for  
16 determination of cytokine concentrations. Concentrations of interleukin-6 were  
17 determined in the supernatant of the PHA stimulated blood, and concentrations of  
18 interferon- $\gamma$  in the supernatant of LPS stimulated blood.

19

20 Details on the determination of micronutrient status indicators were published  
21 earlier (Dijkhuizen *et al.*, 2004). The cytokines interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-6  
22 (IL-6) were measured with ELISA (Pelikine, CLB, Amsterdam, The Netherlands,  
23 calibrated with the standards of the National Institute of Biological Standards and  
24 Controls, Potters Bar, UK). C-reactive protein (CRP) and  $\alpha_1$ -acid glycoprotein  
25 (AGP) were measured using immunoturbidimetric techniques at the Northern Ireland  
26 Centre for Diet Deand Health, University of Ulster, Northern Ireland (Cobas Fara  
27 analyzer, Roche Products, Welwyn, UK). The CV's for the all assays were <10%.

28

29 **Ethical approval.**

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

5

#### 6 **Statistical analysis.**

7           Data were checked for normal distribution using the Kolmogorov-Smirnov  
8 test of normality. Effects of supplementation on number of episodes and days ill were  
9 analysed with ordinal logistic regression analysis, with sex (as factor) and birth  
10 weight and age of the mother (as covariates) included in the model. Duration of each  
11 episode was calculated from total days ill divided by total number of episodes for  
12 each symptom per each child. Episodes were considered distinct from each other  
13 when there was a symptom-free period of at least 7 days between them. Episodes with  
14 more than 1 symptom were considered as 1 episode, but each symptom contributed to  
15 the symptom-specific analysis. The frequencies of reports on 'shortness of breath' and  
16 'vomiting' were too low to allow meaningful statistical analysis as separate  
17 symptoms, but were included in the total illness counts. The occurrence of the first  
18 event for an illness was analysed with Cox regression analysis, again controlling for  
19 sex and birth weight. Cytokine concentrations were expressed as concentrations  
20 relative to those in the control group, by dividing by the mean concentration in the  
21 control group, and were analysed with ANCOVA, controlling for the acute phase  
22 proteins CRP and AGP. If the overall F-test was significant, effects of pre-natal  
23 supplementation was further explored. Main effects were considered significant if  
24  $P < 0.05$ , and interactions (such as between zinc and  $\beta$ -carotene supplementation) was  
25 considered significant if  $P < 0.1$ . However, for all outcomes, the interaction between  
26 prenatal zinc and  $\beta$ -carotene supplementation was not significant ( $P > 0.2$  in all cases)  
27 and therefore, effects of zinc and  $\beta$ -carotene supplementation are presented, and not  
28 the 4 group analyses. All statistical analyses were performed with SPSS (Version  
29 14.0, SPSS Inc, Chicago, USA) software.

30



## 2 RESULTS

3           Of the 179 women who were supplemented during pregnancy until delivery,  
4 170 were enrolled after delivery with their newborn infants for follow-up for 6  
5 months. The infants of nine mothers were not enrolled for follow-up because of twin  
6 pregnancy (n=3), still birth (n=1) or neonatal death (n=5).

7  
8           Newborn infants were evenly born into the 4 groups (Figure 1) between  
9 November 1998 and July 1999. There was no significant difference in birth weight  
10 among the groups. During follow-up, 34 mother-infant pairs dropped out for various  
11 reasons (Figure 1), hence 136 mother-infant pairs completed the study. Compliance to  
12 follow-up visits was excellent, with >94% of the infants having data on 5 or more  
13 visits. All infants received breast feeding, although most of them no longer  
14 exclusively at 6 mo of age.

15  
16           At the age of 6 mo, there were no significant differences in anthropometry  
17 among the groups. As reported earlier, plasma retinol concentrations were  
18 significantly higher in the women supplemented during pregnancy with  $\beta$ -carotene  
19 and zinc combined, whereas  $\beta$ -carotene concentrations were higher in all women who  
20 received  $\beta$ -carotene during pregnancy (Dijkhuizen *et al.*, 2004). Infants born from  
21 mothers receiving  $\beta$ -carotene and zinc combined during pregnancy also had  
22 significantly higher plasma retinol concentrations at 6 mo of age. There were no  
23 differences in plasma zinc concentrations among the groups.

24  
25           Overall, the number of episodes of all illnesses combined was not different  
26 among the groups with a median number of 2 episodes of illness during the first 6 mo  
27 of life (range 0 – 5). Of the infants, 12% (16 infants) was free of any illness during the  
28 first 6 mo, whereas 29.4% (40 infants) reported 1 illness, 23.5% (32 infants) reported  
29 2 illnesses, and 13.9% (19 infants) reported >3 episodes of illness. Girl infants had  
30 significantly fewer episodes of any illness than boy infants (mean 1.6 and 2.3

2 episodes respectively,  $P = 0.003$ ). Also, age of the mother was significantly and  
3 positively correlated to number of illness episodes ( $P=0.004$ ). The median number of  
4 days ill was 6 days (range 0-32 days), and not different among the groups. Again,  
5 girls had significantly fewer days with illness than boys (median 4.0 and 9.0 days  
6 respectively,  $P<0.001$ ) during the first 6 mo of life. This was not only due to fewer  
7 episodes of illness, as girl infants also had significantly shorter duration of episodes  
8 than boy infants, especially of diarrhoea (mean 2.6 and 5.6 days respectively,  
9  $P=0.001$ ) and fever (mean 2.6 and 3.5 days respectively,  $P=0.033$ ).

10

11           Supplementation during pregnancy significantly affected the number of  
12 episodes with cough and diarrhoea during the first 6 mo of life. Infants born from  
13 mothers who received zinc during pregnancy had significantly more episodes of  
14 cough than infants born from mothers not receiving zinc during pregnancy (1.3 and  
15 0.9 episodes respectively,  $P=0.028$ ). In contrast, infants born from mothers receiving  
16 zinc during pregnancy had only half the number of episodes with diarrhoea as  
17 compared to infants born from mothers who did not receive zinc during pregnancy  
18 (means 0.2 and 0.4 episodes respectively,  $P=0.032$ ). This effect of prenatal zinc  
19 supplementation on diarrheal disease was stronger in infants with a lower birth  
20 weight. In infants with a birth weight of  $\leq 3.0$  kg ( $n=61$  infants), prenatal zinc  
21 supplementation was significantly associated with fewer episodes of diarrhoea (mean  
22 0.1 and 0.5 diarrheal episodes respectively,  $P=0.017$ ), whereas in infants with a birth  
23 weight  $>3.0$  kg ( $n=75$ ), there was no significant effect of zinc supplementation during  
24 pregnancy on diarrheal disease (0.3 and 0.4 episodes respectively,  $P>0.1$ ). A cut-off  
25 of 3.0 kg was used, as there were only 11 infants with a birth weight  $<2.5$  kg (8% of  
26 the infants), making meaningful statistical analysis impossible. The duration of each  
27 episode for each symptom was not affected by either zinc or  $\beta$ -carotene  
28 supplementation.

29

2           The time of the first occurrence of illness in the newborn was also affected by  
3 supplementation during pregnancy. Infants born from mothers receiving zinc during  
4 pregnancy had their first episode of diarrhoea significantly later than infants born  
5 from mothers not receiving zinc during pregnancy (Cox regression,  $P=0.026$ ,  
6 controlling for sex,  $\beta$ -carotene supplementation and birth weight, figure 2). In  
7 contrast, infants born from mothers receiving zinc supplementation during pregnancy  
8 had their first episode of cough significantly earlier than infants born from mothers  
9 not receiving zinc during pregnancy (Cox regression,  $P=0.050$ , controlling for sex,  $\beta$ -  
10 carotene supplementation and birth weight, figure 2). There was no significant effect  
11 of  $\beta$ -carotene on the first occurrence of cough or diarrhoea.

12

13           Pre-natal supplementation also significantly affected *ex-vivo* cytokine  
14 production of the infants at 6 mo of age. Infants born from mothers receiving zinc had  
15 a significantly higher interleukin-6 production ( $40.9 \pm 14.7 \mu\text{g/L}$  and  $36.6 \pm 16.5 \mu\text{g/L}$   
16 in the zinc only ( $n=28$ ) and zinc+ $\beta$ -carotene group ( $n= 24$ ) respectively as compared  
17 to  $33.8 \pm 15.3 \mu\text{g/L}$  and  $33.3 \pm 10.4 \mu\text{g/L}$  in the control ( $n=28$ ) and  $\beta$ -carotene ( $n=30$ )  
18 groups). In contrast, infants born from mothers receiving  $\beta$ -carotene had significantly  
19 lower interferon- $\gamma$  production ( $681 \pm 628 \text{ ng/L}$  and  $643 \pm 600 \text{ ng/L}$  in the  $\beta$ -carotene  
20 and  $\beta$ -carotene+zinc groups respectively) as compared to infants born from mother  
21 receiving control ( $966 \pm 939 \text{ ng/L}$ ) or zinc ( $1125 \pm 1083 \text{ ng/L}$ , Figure 3). As  
22 compared to infants born in the control group, zinc supplementation during pregnancy  
23 increased *ex-vivo* IL-6 production at 6 mo of age by 16% ( $P=0.042$ ), but with no  
24 significant effect on IFN- $\gamma$  production (+9%,  $P>0.2$ ). Supplementation with  $\beta$ -  
25 carotene during pregnancy decreased IFN- $\gamma$  production with 36% ( $P=0.030$ ), but had  
26 no significant effect on IL-6 production (-5%,  $P>0.2$ ).

27

## 2 DISCUSSION

3           This study shows that zinc supplementation during pregnancy had a distinct  
4 impact on morbidity and immune function during the first 6 months of life. There  
5 were fewer episodes of diarrhoea, with the first episode occurring later in life, in  
6 infants born from mothers receiving zinc during pregnancy. This effect was stronger  
7 in infants with lower birth weights, which has been reported before (Osendarp *et al.*,  
8 2001). Infants with a lower birth weight are likely to be more at risk for functional  
9 zinc deficiency as catch-up growth requires higher zinc requirements, and hence  
10 would likely benefit more from improved maternal zinc status during pregnancy, even  
11 though this is not reflected in changes in serum zinc concentrations in mother or  
12 infant (King, 1990). At the same time, prenatal zinc supplementation increased the  
13 number of episodes of cough. We believe that the lower incidence of diarrhoea and  
14 the higher incidence of cough are due to the same mechanism: a more reactive  
15 neonatal immune system after prenatal zinc supplementation. The higher incidence of  
16 cough after prenatal zinc supplementation does not necessarily mean more respiratory  
17 infections. It might be that prenatal zinc supplementation leads to a better immune  
18 response to respiratory infection, with more sputum production and more cough,  
19 which is noted by the mother. Illustratively, malnourished children, most of them  
20 severely zinc deficient, have been shown to have pulmonary infections without signs  
21 or symptoms (Doherty *et al.*, 1991).

22

23           The higher production of IL-6 in infants born from mothers receiving zinc  
24 during pregnancy might be due to a better functional zinc status in these infants.  
25 However, anthropometrical indices were not different among the groups, arguing  
26 against differences in functional zinc status at 6 mo of age among the groups. Rather,  
27 we surmise that the prenatal supplementation modified the intra-uterine immune  
28 development of the neonate, explaining the effects found on morbidity and immune  
29 responses at 6 mo of age. Animal models have shown several distinct effects of  
30 marginal zinc deficiency *in utero* on the immune function of the newborn, including

2 reduced amounts of total antibody, reduced T-cell proliferation in response to mitogen  
3 and reduced neutrophil functioning (Wellinghausen, 2001). Interestingly, zinc  
4 supplementation of zinc-deficient pregnant rats increased IFN- $\gamma$  production in pups,  
5 but zinc supplementation of zinc adequate pregnant rats decreased IFN- $\gamma$  production,  
6 highlighting the fine, sensitive balance between insufficient, adequate, and excessive  
7 zinc (Raqib *et al.*, 2007).

8  
9 Human data are lacking however, and to our knowledge, the present study is  
10 the first reporting effects on cytokine production in infants born from mothers  
11 receiving zinc or  $\beta$ -carotene during pregnancy. If the pregnant rat model mentioned  
12 above is representative for humans, the non-significant 9% higher IFN- $\gamma$  production  
13 in the infants born from mothers receiving zinc during pregnancy would argue for  
14 pre-existing, marginal maternal zinc deficiency. The significant reduction in number  
15 of diarrheal disease episodes as found in the present study was also observed in a  
16 study in Bangladesh (Osendarp *et al.*, 2001). In contrast, a study in Peru found no  
17 effect of prenatal zinc supplementation on diarrheal prevalence during the first 12 mo  
18 of life (Iannotti *et al.*, 2008). The amount of zinc supplemented (30 mg in the first two  
19 studies, 15 mg in the study in Peru), as well as the amount of iron (30 mg in the  
20 present study, none in Bangladesh and 60 mg in Peru) may be important factors,  
21 which could explain these differences.

22  
23 Maternal zinc supplementation during pregnancy could give effects on the  
24 immune system which are distinctly different from those of direct supplementation of  
25 the infant. Whether this is due to timing of the intervention in relation to critical  
26 windows in the development of the neonatal immune system *in utero* and after birth,  
27 to other intra-uterine maternal influences, or to differences in dosage is unknown  
28 (West, 2002). However, understanding these differential effects is needed to guide  
29 public health interventions to effectively reduce infant morbidity and mortality  
30 globally. Recently 2 large studies on zinc supplementation in infants and children

2 failed to show a significant benefit of zinc supplementation in children under the age  
3 of 1 year on mortality (Sazawal *et al.*, 2007; Tielsch *et al.*, 2007). Perhaps, maternal  
4 zinc supplementation can be more beneficial in reducing infant mortality.

5

6         Studies on effects of vitamin A supplementation on morbidity and mortality in  
7 infants have given conflicting results. In the present study, despite improved vitamin  
8 A status in the infants in the  $\beta$ -carotene+zinc group, there was no reduction in  
9 morbidity in this group, which corresponds with earlier studies. Moreover, other  
10 immune-modulating events such as childhood vaccinations might modulate the effects  
11 of vitamin A or  $\beta$ -carotene supplementation (Benn *et al.*, 2003). The foetal and  
12 newborn immune response is strongly biased towards Th2-cell cytokine responses.  
13 This is believed to be an evolutionary adaptation, protecting against toxic effects of  
14 Th1-cell cytokines such as IFN- $\gamma$ , which might induce spontaneous abortion (Levy,  
15 2007). However, this Th2-skewing of the immune responses leaves the neonate  
16 vulnerable for bacterial and viral infections. Therefore, beginning directly after birth,  
17 the neonatal immune response is slowly re-calibrated towards a Th1 response, a  
18 process which most infants complete by 6 mo of age (Hertz-Picciotto *et al.*, 2008).  
19 Interestingly, earlier maturation of the Th-1 cell responses has been associated with  
20 protection against allergy and atopic diseases and infections (Levy, 2007). In the  
21 present study, the 36% reduction in IFN- $\gamma$  production after prenatal  $\beta$ -carotene  
22 supplementation warrants further research, as this prolonged bias towards Th2-cell  
23 responses might not only make the infant more susceptible to microbial infections, but  
24 might also predispose to allergy and asthma later in life (Wieringa *et al.*, 2008).  
25 Earlier, we reported lower IFN- $\gamma$  production in infants receiving  $\beta$ -carotene  
26 supplementation or zinc supplementation (Wieringa *et al.*, 2003). In the present study,  
27 only maternal  $\beta$ -carotene supplementation, and not maternal zinc supplementation  
28 affected IFN- $\gamma$  production.

29

2           The present study has several limitations which include the monthly morbidity  
3 recall, and the relatively small sample size. From the 136 infants who finished the  
4 follow-up, cytokine data was available for only 110 of them (81%). This was due to  
5 difficulties in obtaining enough venous blood from the infants for the *ex-vivo*  
6 incubation and due to the fact that sterile conditions were not always met in the field.  
7 For none of the outcomes was there a significant interaction between prenatal  $\beta$ -  
8 carotene supplementation and prenatal zinc supplementation, indicating that effects of  
9 both nutrients are independent of each other.

10

11           Despite these limitations, this study shows that addition of zinc to standard  
12 iron and folic acid supplements can have an impact on the number of diarrheal  
13 episodes during the first 6 mo of life. A recent study from Indonesia showed that  
14 maternal multi-micronutrient supplementation reduced post-natal infant mortality  
15 between 1 and 3 months of age by 30% (Shankar *et al.*, 2008). It is likely that the  
16 effects of prenatal supplementation last much longer than 3 to 6 mo post-partum, and  
17 may even have consequences for diseases in adult life. Therefore, there is an urgent  
18 need to understand the effects of prenatal micronutrient status and supplementation on  
19 neonatal immune response, morbidity and survival to be able to develop interventions  
20 to reduce infant mortality world-wide. At the moment, conflicting evidence from  
21 studies on multiple micronutrients during pregnancy on infant mortality make it hard  
22 for public health policy makers to draw conclusions. For example, in Nepal, addition  
23 of zinc to iron+folic acid supplements nullified the beneficial effect of iron+folic acid  
24 on birth weight (Christian *et al.*, 2003), whereas in China, multiple micronutrient  
25 supplementation during pregnancy improved birth weight, but had no effect on early  
26 neonatal mortality (Zeng *et al.*, 2008). Long-term follow-up of newborns is needed to  
27 understand long-lasting effects of maternal and neonatal nutritional status and  
28 micronutrient supplementation on immune function and health.

29

30

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4 Figure 1. Trial profile.

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6 Figure 2. Survival curves for the first occurrence of diarrhoea, cough and fever in  
7 infants born from mothers supplemented with zinc (-----)  
8 or not (—) during pregnancy. P-values are for Cox-regression analysis, controlling for  
9 sex,  $\beta$ -carotene supplementation and birth weight.

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11 Figure 3. *Ex-vivo* production of IL-6 and IFN- $\gamma$  in infants at 6 mo of age, born from  
12 mothers supplemented during pregnancy with  $\beta$ -carotene and/or zinc, in addition to  
13 iron and folic acid, relative to control group. Bars represent 95% CI  
14

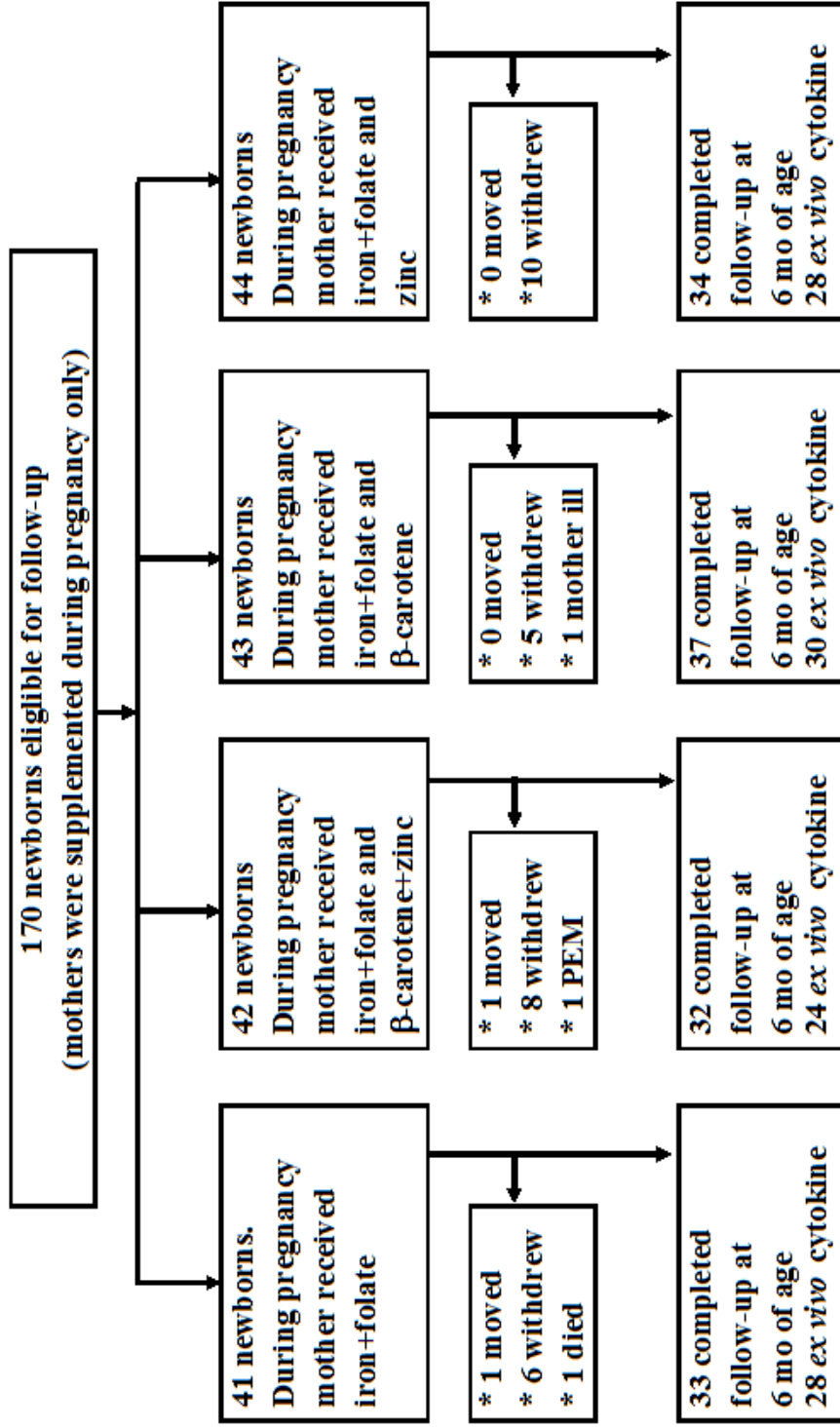


Figure 1. Trial profile

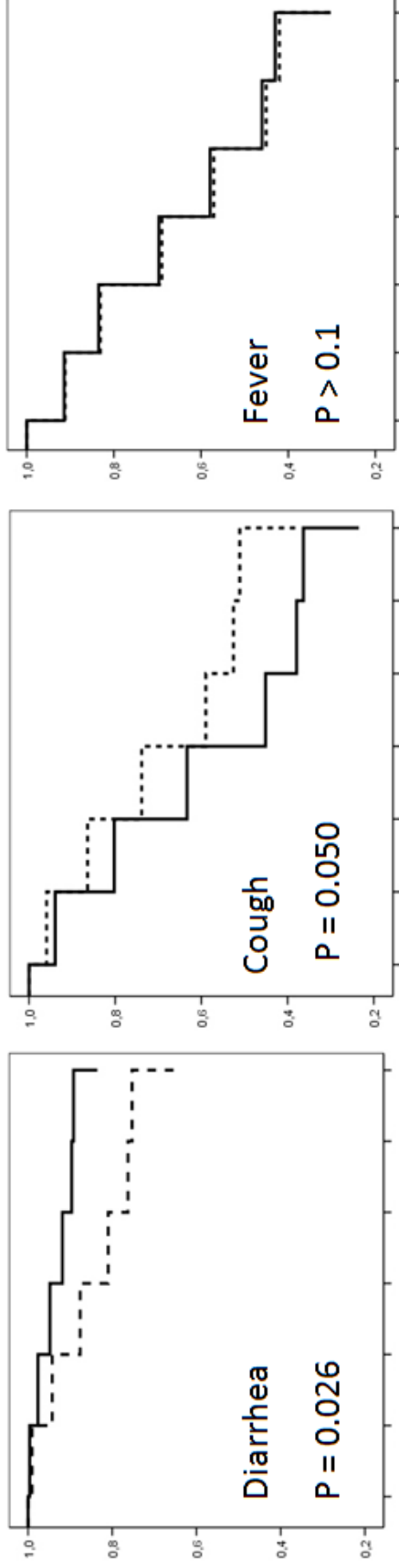


Figure 2. Survival curves for the occurrence of diarrhea, cough and fever in infants born from mothers supplemented with zinc (---) or not (—) during pregnancy. P-values are for Cox-regression analysis, controlling for sex, birth weight and  $\beta$ -carotene supplementation.

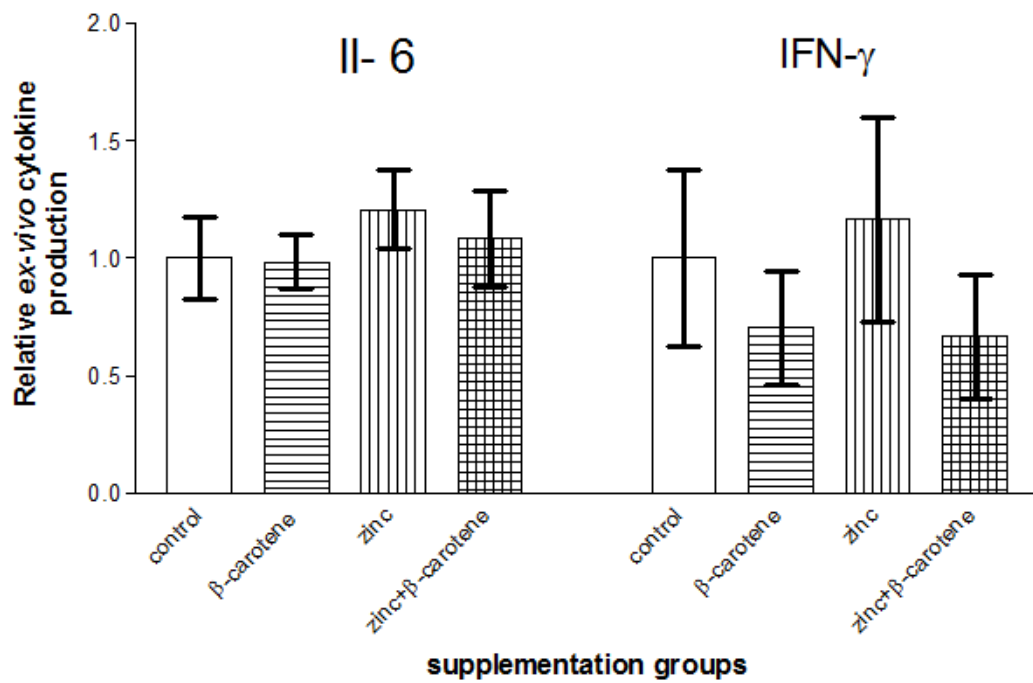


Figure 3. Ex-vivo production of IL-6 and IFN-g in infants at 6 mo of age, born from mothers supplemented during pregnancy with  $\beta$ -carotene and/or zinc, in addition to iron and folic acid relative to control group. Bars represent 95% CI