Iron, zinc, and copper concentrations in breast milk are independent of maternal mineral status[1–3]

Magnus Domellöf, Bo Lönnerdal, Kathryn G Dewey, Roberta J Cohen, and Olle Hernell

ABSTRACT

Background: Little is known about the regulation of iron, zinc, and copper in breast milk and the transport of these minerals across the mammary gland epithelium.

Objective: The objective was to study associations between breast-milk concentrations of iron, zinc, and copper and maternal mineral status.

Design: Milk samples from 191 Swedish and Honduran mothers were collected at 9 mo postpartum. Iron, zinc, and copper concentrations were measured by atomic absorption spectrometry. Blood samples from mothers were analyzed for plasma zinc and copper and 4 indexes of iron status: hemoglobin, plasma ferritin, soluble transferrin receptors, and zinc protoporphyrin. Complementary food energy (CFE) intake was used as an inverse proxy for breast-milk intake.

Results: Mean (±SD) breast-milk concentrations of iron were lower in the Honduran than in the Swedish mothers (0.21 ± 0.25 compared with 0.29 ± 0.21 mg/L; $P < 0.001$), and mean breast-milk concentrations of zinc and copper were higher in the Honduran than in the Swedish mothers [0.70 ± 0.18 compared with 0.46 ± 0.26 mg/L ($P < 0.001$) and 0.16 ± 0.21 compared with 0.12 ± 0.22 mg/L ($P = 0.001$), respectively]. Milk iron was positively correlated with CFE intake ($r = 0.24$, $P = 0.001$) but was not significantly correlated with any iron-status variable. Milk zinc was negatively correlated with CFE intake ($r = -0.24$, $P = 0.001$) but was not significantly correlated with maternal plasma zinc. Milk copper was not significantly correlated with CFE intake or maternal plasma copper.

Conclusions: Milk iron, zinc, and copper concentrations at 9 mo postpartum are not associated with maternal mineral status, which suggests active transport mechanisms in the mammary gland for all 3 minerals. Milk iron concentrations decrease and milk zinc concentrations increase during weaning. *Am J Clin Nutr* 2004; 79:111–5.

KEY WORDS Human milk, breastfeeding, iron, zinc, copper, mammary gland

INTRODUCTION

The World Health Assembly recommends the exclusive breastfeeding of infants until 6 mo of age and continued breastfeeding with appropriate complementary feeding until 2 y of age (1). Iron deficiency and zinc deficiency are public health concerns during infancy, especially in developing countries (2, 3). Iron deficiency in infancy may lead to poor psychomotor development (4, 5), and zinc deficiency may lead to stunted growth (6) and compromised immune function (7). Copper deficiency, as well as copper toxicity, is a concern in infancy, although precise copper requirements have not yet been established for this age group (8). Little is known about the mechanisms regulating the concentrations of iron, zinc, and copper in breast milk (9), although the proteins transporting these minerals across the mammary gland epithelium were recently characterized in cells and in animal studies. Iron is transported by divalent metal transporter 1 through the basolateral membrane into the alveoli and then is exported by ferroportin (also called IREG1) in the apical membrane (W Leong, B Lönnerdal, unpublished observations, 2001). Transferrin receptors are also likely to be involved in iron uptake (10, 11). Zinc uptake by the mammary gland probably occurs via ZTL1, ZIP1, or ZIP4, whereas the export into milk appears to be regulated by ZnT-2 and ZnT-4 (12). Copper uptake by the mammary gland is regulated by Ctr1, a membrane-associated transporter, whereas copper export is regulated by ATP7A (13, 14). The significance of these transporters in the regulation of trace element concentrations in human milk is not yet known.

We previously presented the results from a randomized controlled trial of >200 breastfed infants in Honduras and Sweden who were supplemented with iron; the primary endpoint was iron status at 9 mo (15). These infants were exclusively breastfed until 6 mo and at least partially breastfed to 9 mo. Blood samples were collected from the infants at 9 mo of age for the measurement of various indexes of iron status and for plasma zinc and copper concentrations.

Having access to breast-milk samples from a unique group of lactating mothers with a wide range of nutritional statuses allowed us to investigate whether there are any associations between milk concentrations of iron, zinc, and copper at 9 mo postpartum and maternal iron status or plasma zinc and copper concentrations. This was the aim of the current study. Modern

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indicators of iron status [plasma transferrin receptors (TfR) and zinc protoporphyrin (ZPP)] were included in the assessment of maternal iron status because the relations between these indicators and milk iron have not been investigated. Because socioeconomic conditions in Honduras and Sweden are very different, maternal iron status, zinc status, and possibly copper status were expected to differ between the 2 study sites.

SUBJECTS AND METHODS

Subjects

This study was conducted at 2 sites: San Pedro Sula (Honduras) and Umeå (Sweden) (15). Mother-infant pairs were recruited immediately after birth (in Honduras) or ≈3 mo postpartum (in Sweden). Inclusion criteria were as follows: *1*) gestational age ≥ 37 wk, *2*) birth weight >2500 g, *3*) no chronic illness in the infant, *4*) maternal age ≥16 y, and *5*) infant exclusively breastfed at 4 mo. Between 4 and 6 mo, the mothers were discouraged from giving their infants any other foods or fluids, except for "taste portions" [≤1 Tbsp (15 mL)/d] of foods with little or no iron. Between 6 and 9 mo, the mothers continued breastfeeding but were allowed to give complementary food at their own discretion. No attempt was made by the investigators to influence the choice of foods or the extent of breastfeeding. The study was approved by the Human Subjects Review Committee of the University of California, Davis, and the Ethical Committee, Faculty of Medicine and Odontology, Umeå University, Sweden. All participating mothers gave written informed consent.

Data collection and analysis

Breast-milk samples (10–40 mL) were collected at 9 mo postpartum. Samples were collected in the morning ≥1 h after the previous breastfeeding. Breast milk was expressed by hand or manual pump from one breast into plastic containers provided by the investigators. Single-use plastic containers were tested and found negative for mineral contamination. Plastic pumps were washed in 0.1% nitric acid before being reused and were tested and found negative for mineral contamination after being washed. Samples were transferred to zinc-free, screw-cap, plastic tubes; frozen at −20 °C; and transported to the University of California, Davis, for analysis. Milk samples were wet ashed and analyzed according to Clegg et al (16) with some minor modifications. Samples were thawed, mixed thoroughly, and digested in ultrapure concentrated nitric acid (Fisher, Los Angeles) at room temperature for 96 h and then at subboiling temperature for 6–9 h. Iron, zinc, and copper concentrations were determined by atomic absorption spectrometry (model Smith-Heifjie 4000; Thermo Jarrell Ash Corporation, Franklin, MA). Elemental standards from Fisher Scientific International Inc (Los Angeles) and standard reference materials from the National Institute of Standards and Technology (Gaithersburg, MD) were used. The analyzed reference values were within 95–105% of the certified value.

Venous blood (≈5 mL) was obtained from the mothers at 9 mo postpartum. In Honduras, maternal blood samples were generally obtained in the morning after an overnight fast. In Sweden, the samples were generally taken in the morning, but mothers were not necessarily fasting. Part of the sample was collected in an EDTA-treated test tube and immediately analyzed in duplicate for hemoglobin (HemoCue, Ängelholm, Sweden) and ZPP (Protofluor Z; Helena Labs, Beaumont, TX). These 2 instruments were checked weekly against standard solutions at both sites. The other part of the sample was collected in a lithium heparin–treated tube and, after centrifugation (1750 × *g*, 10 min, room temperature), plasma was stored frozen at −20 °C until analyzed for ferritin (Coat-A-Count Ferritin IRMA; Diagnostic Products Corp, Los Angeles) and TfR (Ramco, Houston). Plasma samples were diluted 1:5 (by vol) in 1% nitric acid, the samples were allowed to undergo digestion for 2 d, and concentrations of zinc and copper were determined by atomic absorption spectrometry as described above.

Complementary food intakes between 6 and 9 mo were estimated in Honduras by a biweekly 24-h dietary recall and a food-frequency questionnaire and in Sweden by a monthly 5-d food diary. Nutrient intakes in Honduras were calculated by using the FOOD PROCESSOR program (ESHA Research, Salem, OR), and those in Sweden were calculated by using food-composition tables from the National Food Administration and information from Swedish baby food manufacturers. Infant weight at 9 mo of age was measured by the investigators.

Statistics

All statistical analyses were performed by using SPSS software version 11.0 (SPSS Inc, Chicago). Student's *t* test was used for comparing means, Fisher's exact test for comparing proportions, linear regression analysis for studying correlations, and analysis of covariance for studying correlations while potential confounders were controlled for.

Because the distributions of plasma ferritin, ZPP, milk iron, milk zinc, milk copper, and complementary food energy intake were skewed, these variables were log transformed in all calculations. For presentation, the variables were transformed back to the original scale and presented as geometric means. To assess the possible predictors of milk mineral concentrations, multiple regression analyses were performed, including the following explanatory variables: *1*) maternal mineral status (plasma zinc and copper or an index of iron status), *2*) complementary food energy intake (inverse proxy for breast-milk intake), and *3*) study site (Honduras or Sweden).

RESULTS

Of the 263 mother-infant pairs recruited at 4 mo postpartum, 214 remained in the study at 9 mo postpartum. The total dropout rate was not significantly different by study site. Considering both study sites separately, there were no significant differences between dropouts and nondropouts with respect to maternal age, weight, height, parity, infant sex, or birth weight. The most common reasons for dropout were suspected side effects of the iron or placebo drops ($n = 12$) and the family moving out of the study area ($n = 8$). Of the mothers remaining in the study at 9 mo, 21 could not produce a sufficient breast-milk sample (10 mL) and 2 refused blood sampling. Sufficient breast-milk and blood samples were obtained from 191 mothers, and data from these subjects were included in the statistical analysis.

Compared with the Swedish mothers, the Honduran mothers were significantly younger, were of higher parity, and had lower weights and heights, and their infants had lower weights

TABLE 1
Background characteristics of the subjects by site[1]

	Honduras (n = 105)	Sweden (n = 86)
Maternal age (y)	26 ± 6	31 ± 4[2]
Parity (n)	2.8 ± 1.9	1.8 ± 0.9[2]
Maternal weight (kg)	56 ± 11	65 ± 10[2]
Maternal height (cm)	151 ± 6	166 ± 6[2]
Birth weight (kg)	3.2 ± 0.4	3.6 ± 0.4[2]
Birth length (cm)	49 ± 2	51 ± 2[2]

[1] $\bar{x}$ ± SD.
[2] Significantly different from Honduras, $P < 0.001$ (two-tailed t test, independent samples).

and lengths at birth (**Table 1**). The mean (±SD) complementary food energy intake of infants was lower in Honduras than in Sweden (25 ± 11 compared with 41 ± 22 kcal · kg^{-1} · d^{-1}; $P < 0.001$). There was a significantly higher proportion of Honduran than of Swedish mothers with low hemoglobin (12% compared with 0%), low ferritin (32% compared with 12%), and high ZPP (6% compared with 0%), whereas the proportions of mothers with high TfR concentrations were not significantly different between sites (5% compared with 4%) (**Table 2**). Infant birth weight, maternal age, parity, weight, and height were not significantly correlated with any of the main outcome variables when site was adjusted for. Therefore, these variables were not included as potential confounders in subsequent analyses.

The Honduran mothers had significantly lower breast-milk iron concentrations than did the Swedish mothers (**Table 3**). After control for study site and complementary food energy intake, no significant correlations between milk iron concentration and any of the indexes of maternal iron status were observed: hemoglobin ($r = -0.03$, $P = 0.73$), ferritin ($r = -0.06$, $P = 0.41$), ZPP ($r = 0.00$, $P = 0.99$), and TfR ($r = -0.05$, $P = 0.44$). Furthermore, there was no significant difference in the mean milk iron concentration between anemic and nonanemic Honduran mothers (0.26 compared with 0.21 mg/L; $P = 0.23$). However, there was a positive correlation between milk iron concentration and complementary food energy intake ($r = 0.24$, $P = 0.001$) after control for the study site, which suggests that milk iron concentrations increase with weaning. When the 2 study sites were analyzed separately, no significant correlations in Sweden or Honduras between milk iron concentration and any of the iron- status variables were observed after control for complementary food energy intake. In Honduras, there was a positive correlation between milk iron concentration and complementary food energy intake ($r = 0.30$, $P = 0.002$). This correlation was not significant in the Swedish subsample ($r = 0.17$, $P = 0.116$).

TABLE 2
Proportion of mothers fulfilling criteria for iron deficiency by site

Deficiency cutoff	Honduras (n = 105)	Sweden (n = 86)
	%	
Hemoglobin < 120 g/L	12	0[1]
Ferritin < 12 μg/L	32	12[1]
Zinc protoporphyrin > 80 μmol/mol heme	6	0[2]
Transferrin receptor > 8.3 mg/L	5	4

[1,2] Significantly different from Honduras (Fisher's exact test, two-tailed): [1] $P = 0.001$, [2] $P = 0.033$.

TABLE 3
Iron, zinc, and copper concentrations in the breast milk of mothers by site[1]

	Honduras (n = 105)	Sweden (n = 86)
	mg/L	
Iron	0.21 ± 0.25	0.29 ± 0.21[2]
Zinc	0.70 ± 0.18	0.46 ± 0.26[2]
Copper	0.16 ± 0.21	0.12 ± 0.22[3]

[1] Geometric $\bar{x}$ ± SD.
[2,3] Significantly different from Honduras (two-tailed t test, independent samples): [2] $P < 0.001$, [3] $P = 0.001$.

The Honduran mothers had significantly higher breast-milk zinc concentrations than did the Swedish mothers (Table 3). After control for study site and complementary food energy intake, there was no significant correlation between milk zinc concentration and maternal plasma zinc ($r = -0.06$, $P = 0.61$). After control for study site, milk zinc concentration was negatively correlated with complementary food energy intake ($r = -0.24$, $P = 0.001$), which suggests that milk zinc concentrations decrease with weaning. When the 2 study sites were analyzed separately, no significant correlation in Sweden or Honduras between milk zinc concentration and maternal plasma zinc was observed after control for complementary food energy intake. In Sweden, there was a negative correlation between milk zinc concentration and complementary food energy intake ($r = -0.27$, $P = 0.012$). This correlation was not significant in the Honduran subsample ($r = -0.19$, $P = 0.058$).

The Honduran mothers had significantly higher breast-milk copper concentrations than did the Swedish mothers (Table 3). In the multivariate analysis that included maternal plasma copper, complementary food energy intake, and study site as explanatory variables, there was no significant correlation between milk copper concentration and maternal plasma copper ($r = 0.12$, $P = 0.08$) or between milk copper concentration and complementary food energy intake ($r = 0.14$, $P = 0.069$). When the 2 study sites were analyzed separately, there was no significant correlation in Sweden or Honduras between milk copper concentration and maternal plasma copper, after control for complementary food energy intake. In Honduras, a positive correlation between milk copper concentration and complementary food energy intake ($r = 0.24$, $P = 0.012$) was observed. This correlation was not observed in the Swedish subsample ($r = 0.03$, $P = 0.76$).

DISCUSSION

Previous studies have shown no correlation between maternal iron status and milk iron concentration (17, 18). Furthermore, maternal dietary iron appears to have little effect on human milk iron concentration (19). A single study has shown increased concentrations of iron in the milk from severely anemic mothers (20). Unlike previous studies, the current study included a larger number of lactating mothers, a wider range of maternal nutritional status, and an analysis of TfR, which is a

novel indicator of iron status. As found in most previous studies, we found no correlation between milk iron and any of the iron-status variables (hemoglobin, ferritin, ZPP, and TfR). Our results suggest that the site difference in milk iron concentrations (lower in Honduras) was caused by differences in milk volume (higher in Honduras) rather than by differences in maternal iron status.

Our observation that milk zinc concentrations were >50% higher in Honduras than in Sweden was unexpected because the Honduran mothers had significantly lower plasma zinc concentrations. However, the multivariate analysis showed that the site difference in milk zinc concentration was attributable to differences in milk volume rather than to differences in maternal plasma zinc. This finding is consistent with the observation by Moser et al (21) of similar milk zinc concentrations in Nepali and American women despite lower plasma zinc concentrations in the Nepali women. It is well known that plasma zinc concentrations decrease after a meal (22). In Honduras, maternal blood samples were obtained in the morning after an overnight fast. In Sweden, the samples were generally taken in the morning, but mothers were not necessarily fasting. This difference in study procedure is not likely to explain the differences in plasma zinc between study sites, because it would have resulted in higher rather than lower plasma zinc concentrations in Honduras.

It is interesting that prolonged breastfeeding and low complementary food intake in Honduras were associated with higher zinc concentrations in breast milk from Honduran women, despite lower maternal plasma zinc concentrations. This may be an important factor in the prevention of zinc deficiency among breastfed infants in developing countries. Most previous studies found no correlation between maternal dietary zinc intake and milk zinc concentration (19, 23). A single study showed a slight increase in milk zinc after zinc supplementation (24), but a subsequent, larger study by the same researchers showed no effect on milk zinc (25). However, it is likely that most of the women in those studies had adequate zinc status. There are few human studies on milk zinc in women with low zinc status, although this has been explored in animal studies. When pregnant and lactating rats were fed a diet marginal in zinc, no effect on milk zinc was found (26). In lactating rats with marginal zinc deficiency, we found significant up-regulation of the expression of ZnT-2 and ZnT-4, which would be a compensatory mechanism to maintain milk zinc concentrations (12).

Previous studies found no effect of dietary copper on the copper concentration of human milk (19). In our study, we found no correlation between maternal plasma copper and milk copper. It is interesting that milk copper was higher in the Honduran than in the Swedish women. We found that milk copper was significantly elevated in zinc-deficient lactating rats and that this increase was associated with increased expression of Ctr1 and ATP7A (12). The mechanism behind this up-regulation of milk copper transport during zinc deficiency is not yet known. The observed site difference in milk copper (higher in Honduras) cannot be explained by the observed positive correlation between complementary food energy intake (lower in Honduras) and milk copper concentration in the Honduran subsample. We conclude that milk copper concentrations are determined by factors other than maternal plasma copper and milk volume.

If the passage of iron, zinc, and copper from plasma to milk in the mammary gland were the result of passive diffusion, positive correlations would be expected between plasma mineral status (concentration) and milk mineral concentration. Because we found no such correlations, our results suggest a regulated transport of iron, zinc, and copper through the mammary gland epithelium. The findings of recent animal studies agree with such an interpretation (12).

The concentrations of some nutrients in breast milk (eg, fat, protein, and sodium) increase during weaning, whereas others (eg, lactose and calcium) decrease (27). We found that a high complementary food energy intake (weaning) was associated with low milk zinc and high milk iron concentrations, which suggests that these 2 minerals are affected differently by weaning. This finding is supported by a previous study by our group in which breast-milk concentrations of zinc decreased and those of iron increased in late lactation (27). Decreased milk zinc and increased milk iron with increasing complementary food energy intake result in a decreased ratio of the concentrations of zinc and iron in breast milk during the process of weaning. The consequences of this change in ratio, if any, are unknown. We speculate that the increase in milk iron during weaning is explained by the fact that milk protein concentrations increase during weaning, and a large proportion of iron is bound to one of the milk proteins, namely lactoferrin (28). Milk zinc, however, is found largely in the low-molecular-weight fraction (associated with citrate), the concentration of which is associated with lactose and water transport, both of which decrease during weaning (28). Further studies of mammary gland physiology during weaning are needed to explain these observations.

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RJC collected the Honduran data, and MD collected the Swedish data. BL was responsible for all of the laboratory analyses. MD analyzed the data. MD, BL, KGD, and OH were responsible for the study design and the preparation of the manuscript. None of the authors had a conflict of interest to report.

REFERENCES

1. World Health Assembly (WHA). Infant and young child nutrition. Resolution WHA 54.2 2001.
2. Domellöf M, Hernell O. Iron-deficiency anaemia during the first two years of life. Scand J Nutr 2002;46:20–30.
3. Hambidge KM. Zinc deficiency in young children. Am J Clin Nutr 1997;65:160–1.
4. Roncagliolo M, Garrido M, Walter T, Peirano P, Lozoff B. Evidence of altered central nervous system development in infants with iron deficiency anemia at 6 mo: delayed maturation of auditory brainstem responses. Am J Clin Nutr 1998;68:683–90.
5. Idjradinata P, Pollitt E. Reversal of developmental delays in iron-deficient anaemic infants treated with iron. Lancet 1993;341:1–4.
6. Rivera JA, Ruel MT, Santizo MC, Lönnerdal B, Brown KH. Zinc supplementation improves the growth of stunted rural Guatemalan infants. J Nutr 1998;128:556–62.
7. Ruel MT, Rivera JA, Santizo MC, Lönnerdal B, Brown KH. Impact of zinc supplementation on morbidity from diarrhea and respiratory infections among rural Guatemalan children. Pediatrics 1997;99:808–13.

8. Lönnerdal B. Copper nutrition during infancy and childhood. Am J Clin Nutr 1998;67(suppl):1046S–53S.
9. Lönnerdal B. Regulation of mineral and trace elements in human milk: exogenous and endogenous factors. Nutr Rev 2000;58:223–9.
10. Sigman M, Lönnerdal B. Response of rat mammary gland transferrin receptors to maternal dietary iron during pregnancy and lactation. Am J Clin Nutr 1990;52:446–50.
11. Sigman M, Lönnerdal B. Relationship of milk iron and the changing concentration of mammary tissue transferrin receptors during the course of lactation. J Nutr Biochem 1990;1:572–6.
12. Kelleher SL, Lönnerdal B. Long-term marginal intakes of zinc and retinol affect retinol homeostasis without compromising circulating levels during lactation in rats. J Nutr 2001;131:3237–42.
13. Ackland ML, Anikijenko P, Michalczyk A, Mercer JF. Expression of Menkes copper-transporting ATPase, MNK, in the lactating human breast: possible role in copper transport into milk. J Histochem Cytochem 1999;47:1553–62.
14. Kelleher SL, Lonnerdal B. Zinc transporters in the rat mammary gland respond to marginal zinc and vitamin A intakes during lactation. J Nutr 2002;132:3280–5.
15. Domellöf M, Cohen RJ, Dewey KG, Hernell O, Rivera LL, Lönnerdal B. Iron supplementation of breast-fed Honduran and Swedish infants from 4 to 9 months of age. J Pediatr 2001;138:679–87.
16. Clegg MS, Keen CL, Lönnerdal B, Hurley LS. Influence of ashing techniques on the concentration of trace elements in animal tissues. 1: Wet ashing. Biol Trace Elem Res 1981;3:107–15.
17. Murray MJ, Murray AB, Murray NJ, Murray MB. The effect of iron status of Nigerian mothers on that of their infants at birth and 6 months, and on the concentration of Fe in breast milk. Br J Nutr 1978;39:627–30.
18. Celada A, Busset R, Gutierrez J, Herreros V. No correlation between iron concentration in breast milk and maternal iron stores. Helv Paediatr Acta 1982;37:11–6.
19. Lönnerdal B. Effects of maternal dietary intake on human milk composition. J Nutr 1986;116:499–513.
20. Fransson GB, Agarwal KN, Gebre MM, Hambraeus L. Increased breast milk iron in severe maternal anemia: physiological "trapping" or leakage? Acta Paediatr Scand 1985;74:290–1.
21. Moser PB, Reynolds RD, Acharya S, Howard MP, Andon MB, Lewis SA. Copper, iron, zinc, and selenium dietary intake and status of Nepalese lactating women and their breast-fed infants. Am J Clin Nutr 1988;47:729–34.
22. Wallock LM, King JC, Hambidge KM, English-Westcott JE, Pritts J. Meal-induced changes in plasma, erythrocyte, and urinary zinc concentrations in adult women. Am J Clin Nutr 1993;58:695–701.
23. Moser PB, Reynolds RD. Dietary zinc intake and zinc concentrations of plasma, erythrocytes, and breast milk in antepartum and postpartum lactating and nonlactating women: a longitudinal study. Am J Clin Nutr 1983;38:101–8.
24. Krebs NF, Hambidge KM, Jacobs MA, Rasbach JO. The effects of a dietary zinc supplement during lactation on longitudinal changes in maternal zinc status and milk zinc concentrations. Am J Clin Nutr 1985;41:560–70.
25. Krebs NF, Reidinger CJ, Hartley S, Robertson AD, Hambidge KM. Zinc supplementation during lactation: effects on maternal status and milk zinc concentrations. Am J Clin Nutr 1995;61:1030–6.
26. Beshgetoor D, Lönnerdal B. Effect of marginal maternal zinc deficiency in rats on mammary gland zinc metabolism. J Nutr Biochem 1997;8:573–8.
27. Dewey KG, Finley DA, Lönnerdal B. Breast milk volume and composition during late lactation (7–20 months). J Pediatr Gastroenterol Nutr 1984;3:713–20.
28. Neville MC, Allen JC, Archer PC, et al. Studies in human lactation: milk volume and nutrient composition during weaning and lactogenesis. Am J Clin Nutr 1991;54:81–92.

Folic acid fortification in the prevention of neural tube defects

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Dear Sir:

We believe that Clarke and Grimley Evans (1) are incorrect in 3 important ways when they argue against the immediate introduction of universal fortification of flour with folic acid to prevent neural tube defects in the United Kingdom.

First, there is no evidence that fortification at the level recommended by the Committee on Medical Aspects of Food and Nutrition Policy (COMA) will mask the diagnosis of vitamin B-12 deficiency. Clarke and Grimley Evans overlook recent evidence indicating that folic acid fortification in the United States has not altered the presentation of vitamin B-12 deficiency (2). Their arguments, which suggest that vitamin B-12 neuropathy will increase after folic acid fortification, are therefore without foundation and are not supported by any evidence. In maintaining their proposition of masking, they are preventing the introduction of a beneficial public health measure without good reason or justification.

Second, Clarke and Grimley Evans are incorrect in stating that the UK COMA recommendation that flour in the United Kingdom should be fortified with folic acid at the level of 240 μg/100 g flour was conditional on fortification being set at a minimal level so that the level could never be exceeded—a totally impractical condition—or that fortification be delayed until a "means be instituted to identify and protect elderly persons who are vitamin deficient." Two of us (NJW and AVH) were members of the COMA working group and we are surprised at the statement by Clarke and Grimley Evans because Grimley Evans was himself Chairman of the Working Group that produced the report. A review of the report confirms that although these issues were considered, the recommendation for folic acid fortification was not made conditional.

Third, Clarke and Grimley Evans are incorrect in advising against folic acid fortification until a screening program for vitamin B-12 deficiency is introduced. Vitamin B-12 deficiency may be a public health problem, although this needs to be assessed by determining the incidence of the clinical disease it causes rather than by determining the prevalence of serum vitamin B-12 concentrations below specified cutoffs or the prevalence of homocysteine concentrations above specified cutoffs. The public health solution to any vitamin B-12 deficiency problem is to directly address the problem and not to use it to stop the remedy of another public health problem (neural tube defects) that can be substantially prevented through folic acid fortification. Screening for vitamin B-12 deficiency, which they propose, would be costly and probably of limited efficacy because few screening programs achieve 100% coverage. A preferred solution to vitamin B-12 deficiency in the elderly would be to fortify the diet with vitamin B-12. However, this issue should be separate from the issue of food fortification with folic acid. Whatever is done about vitamin B-12 deficiency, this should not be a reason for failing to prevent neural tube defects through folic acid fortification—a policy that has been accepted by 38 countries throughout the world. One public health measure should not hold up another.

REFERENCES

1. Clarke R, Grimley Evans J. Reply to NJ Wald et al. Am J Clin Nutr 2004;79:338–9.
2. Mills JL, Von Kohorn I, Conley MR, et al. Low vitamin B-12 concentrations in patients without anemia: the effect of folic acid fortification of grain. Am J Clin Nutr 2003;77:1474–7.

Reply to NJ Wald et al

Dear Sir:

We reject all 3 accusations of error made by Wald, Law, and Hoffbrand. Where there is even a remote possibility of harm from a public health intervention, the burden of proof lies with those who choose to assert that harm could not result. What has happened in the United States at one level of folic acid fortification is not necessarily helpful in predicting what might happen in the United Kingdom at a higher level and where the medical condition of older people, including their vitamin B-12 status, may be less well documented.

Wald et al are incorrect in asserting that the Committee on Medical Aspects of Food and Nutrition Policy (COMA) Report (1) made a "recommendation" about fortification. For reasons set out in the preface to the Report, recommendations would have been improper; the Committee's task was to appraise options. The modeling that was set out in the Report evaluated several possible levels of fortification as targets to be attained but not exceeded (paragraph 9.5.10). For each target, the number of neural tube defects likely to be prevented and the proportion of older people exposed to total daily folate intakes in excess of 1 mg were estimated. There would have been no sense in calculating the latter values if the Committee had expected fortification levels to be treated as minima or as merely approximate.

The need to protect older people with undiagnosed vitamin B-12 deficiency as part of "any general policy to increase dietary folic acid consumption" is clearly and repeatedly expressed in the Report. We

and others have been exploring ways and means of implementing such protection. We anticipate that sufficient data will soon be available to develop a rational approach to the issue in the United Kingdom, so that the 2 public health measures alluded to in Wald et al's final sentence could go forward in concert.

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REFERENCE

1. Committee on Medical Aspects of Food and Nutrition Policy. Folic acid and the prevention of disease. London: The Stationery Office, 2000.

Hypovitaminosis D is associated with insulin resistance and β cell dysfunction

Dear Sir:

It was interesting to see the elegant explanation by Chiu et al (1) of associations between hypovitaminosis D and both insulin resistance and β cell dysfunction in several different ethic groups, which supports the idea of hypovitaminosis vitamin D as a risk factor for the metabolic syndrome, including Type 2 diabetes (2). Their findings are of particular interest because their subjects were normoglycemic, whereas our earlier data, quoted by Chiu et al, came from dysglycemic subjects (3, 4). The additional finding of adverse effects of hypovitaminosis D on the fasting lipid profile is also important in view of the reports of increased risk of ischemic heart disease with vitamin D deficiency in cross-sectional studies in communities where vitamin D deficiency is common (5, 6). In 146 healthy, nondiabetic British subjects originally from an area of Bangladesh (Sylhet) where both soluble C-reactive protein concentrations and plasma metalloproteinase 9 concentrations were inversely related to vitamin D status (7), we now find on univariate analysis (unpublished data) that serum 25-hydroxyvitamin D concentration relates directly to total and LDL cholesterol and to both apolipoproteins A1 (apo A1) and B (apo B) (**Table 1**). However, when we examined these data by using the same variables as in the study by Chiu et al, except ethnicity, we found serum 25-hydroxyvitamin D concentrations to relate directly to both total and HDL cholesterol and to apo A1 and apo B but to relate inversely to triacylglycerol. On reexamination that includes additional variables such as smoking, however, serum 25-hydroxyvitamin D concentrations appear to be an independent predictor of increases in apo A1 alone.

In the report of Chiu et al, ethnicity was not a predictor of any of the lipid profile variables assessed, although it was a predictor for vitamin D status (1). In view of our findings, however, we wonder whether there may be any variation in the relations of serum 25(OH)D concentration to elements of the fasting lipid profile between the ethnic groups examined.

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REFERENCES

1. Chiu KC, Chu A, Go VLW, Saad MF. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. Am J Clin Nutr 2004;79:820–5.
2. Boucher BJ. Inadequate vitamin D status: does it contribute to the disorders comprising 'syndrome X'? Br J Nutr 1998;79:315–27.
3. Boucher BJ, Mannan N, Noonan K, Hales CN, Evans SJW. Glucose intolerance and impairment of insulin secretion in relation to vitamin D deficiency in East London Asians. Diabetology 1995;38:1239–45.
4. Baynes KC, Boucher BJ, Feskens EJ, Kromhout D. Vitamin D, glucose tolerance and insulinaemia in elderly men. Diabetologia 1997;40:344–7.
5. Scragg R, Jackson R, Holdaway IM, Lim T, Beaglehold R. Myocardial infarction is inversely related with plasma 25-hydroxyvitamin D levels: a community-based study. Int J Epidemiol 1990;19:559–63.
6. Grimes DS, Hindle E, Dyer T. Sunlight, cholesterol and coronary heart disease. Q J Med 1996;89:579–89.
7. Timms PM, Mannan N, Hitman GA, et al. Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? QJM 2002;95:787–96.

Reply to BJ Boucher et al

Dear Sir:

We appreciate the comments from Boucher et al regarding our recent article (1), specifically with respect to the issue of a relation between serum 25-hydroxyvitamin D concentrations and the lipid profile. Because elevated C-reactive protein concentrations are associated with insulin resistance and the presence of the metabolic syndrome (2), the published data of Timms et al (3) on the relation between hypovitaminosis D and elevated C-reactive protein

TABLE 1
Regression analysis of the effect of 25-hydroxyvitamin D on fasting lipid profile in the 56 whites in the study

	Univariate analysis		Multivariate analysis[1]	
	R	*P*	*r*	*P*
Triacyglycerols	−0.1685	NS	—	NS
Total cholesterol	−0.4125	0.0019	−0.3489	0.0038
HDL cholesterol	0.0801	NS	—	NS
LDL cholesterol	−0.4169	0.0017	−0.2866	0.0279

[1] Covariates considered were sex, age, season, systolic and diastolic blood pressure, BMI, waist-hip ratio, and 25-hydroxyvitamin D.

($r = -0.22$, $P = 0.031$) in a sample of 146 subjects are in accord with our observation that hypovitaminosis D is associated with insulin resistance and the metabolic syndrome (1). Their unpublished observation of a positive correlation of serum 25-hydroxyvitamin D with both total cholesterol and apolipoprotein B and a negative correlation with triacylglycerols does not support the possibility of a relation between hypovitaminosis D and insulin resistance phenotypes.

We have no information regarding the concentrations of C-reactive protein, plasma metalloproteinase 9, and apoliproteins A1 and B in this sample. Because there were only 126 subjects in our multiracial sample, we focused on the whites, who were the largest racial or ethnic group in the sample ($n = 56$). The influence of 25-hydroxyvitamin D on fasting lipid profile is shown in **Table 1**. The results are highly similar to our published results (1). The negative association of serum 25-hydroxyvitamin D with both total and LDL cholesterol is in agreement with the association of hypovitaminosis D with insulin resistance and the metabolic syndrome. The 3 nonwhite ethnic groups in our sample were not large enough to have adequate power to detect the association. Although the association study provides no information on the cause-effect relation, hypovitaminosis D could be one of the culprits of insulin resistance.

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REFERENCES

1. Chiu KC, Chu A, Go VL, Saad MF. Hypovitaminosis D is associated with insulin resistance and β cell dysfunction. Am J Clin Nutr 2004;79:820–5.
2. Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB Sr, Wilson PW. C-reactive protein, the metabolic syndrome, and prediction of cardiovascular events in the Framingham Offspring Study. Circulation 2004;110:380–5.
3. Timms PM, Mannan N, Hitman GA, et al. Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders? QJM 2002;95:787–96.

The single nucleotide polymorphism (80G→A) of reduced folate carrier gene in trisomy 21

Dear Sir:

The reduced folate carrier gene (*RFC1* or *SLC19A1*) located on chromosome 21 (21q22.3) codes for the reduced folate carrier, which is responsible for 5-methyltetyrahydrofolate internalization within cells. We published earlier the 80G→A single-nucleotide polymorphism (SNP) in human reduced folate carrier cDNA and hypothesized that it influences folate metabolism or the plasma total homocysteine concentration (1). The SNP 80G→A polymorphism (rs1051266; http://www.ncbi.nlm.nih.gov at the SNP database) is a guanine-to-adenine exchange at nucleotide 80 in *RFC1* cDNA. Because of the extra copy of chromosome 21 in persons with trisomy 21, or Down syndrome, a dosage effect of *RFC1* and a functional effect of the SNP 80G→A polymorphism may contribute to a imbalance of one-carbon-derived metabolites and DNA methylation. Allelic ratios are 3:0, 2:1, 1:2, and 0:3 for the *80GGG*, *80GGA*, *80GAA*, and *80AAA* genotypes, respectively, in trisomy 21 compared with 2:0, 1:1, and 0:2 for the *80GG*, *80GA*, *80AA* genotypes, respectively, in control subjects.

In a study published in this issue of the Journal, we analyzed the 80G→A genotype distribution in 160 persons (87 men and 73 women) aged 26 ± 4 y ($\bar{x}$ ± SD; range: 15–46 y) with full trisomy 21 confirmed by karyotype and in 160 healthy, unrelated control subjects (2). With the standard restriction fragment length polymorphism method using the *Hha*I (or *Cfo*I) restriction enzyme (1, 3), we were not able to distinguish heteroallelic individuals containing 1 or 2 copies of each allele among the persons with Down syndrome. We have now applied the pyrosequencing technology (4) to determine SNP genotypes in persons with trisomy 21.

The principle of pyrosequencing is based on the polymerization of single-stranded amplified DNA fragments and the detection of de novo incorporation of nucleotides, which leads to the generation of visible light in proportion to the number of incorporated nucleotides (**Figure 1**). Briefly, DNA samples were amplified by polymerase chain reaction with the sense primer HsRFC 4.3 5′-TGC AGA CCA TCT TCC AAG G-3′ and the antisense primer HsRFC 4.3 5′-CCA TGA AGC CGT AGA AGC-3′. The amplified DNA samples were purified by using streptavidin-Sepharose HP beads (Amersham Biosciences, Orsay, France) and a pyrosequencing sample preparation kit (Pyrosequencing AB, Uppsala, Sweden) according to the manufacturer's instructions. Purified samples were run on a PSQ 96MA instrument containing a pyrosequencing cartridge filled with dATPαS, dTTP, dCTP, dGTP, substrate, and enzyme as supplied in a PSQ reagent kit (Pyrosequenring). The 4 nucleotides are added stepwise to the primed DNA template. Analysis of sequences was performed automatically by the ALLELE QUANTIFICATION software (Pyrosequencing). The intensity of the light signal is directly proportional to the number of nucleotides incorporated. Genotypes were determined by comparison of the peak heights of allele positions with the theoretical results predicted by the software.

The pyrosequencing results of our initial samples (2) show that among the persons with trisomy 21, 37 persons (23.7%) were homozygous *80GGG*, 52 persons (33.5%) were heterozygous *80GGA*, 44 persons (28.4%) were heterozygous *80GAA*, and 22 persons (14.2%) were homozygous *80AAA*. Among the control subjects, the numbers of subjects were as follows: 53 persons (33.4.%) were homozygous *80GG*, 74 persons (46.5%) were heterozygous *80GA*, and 32 persons (20.0%) were homozygous *80AA*. In conclusion, genotyping SNP and determination of allele frequency in trisomy

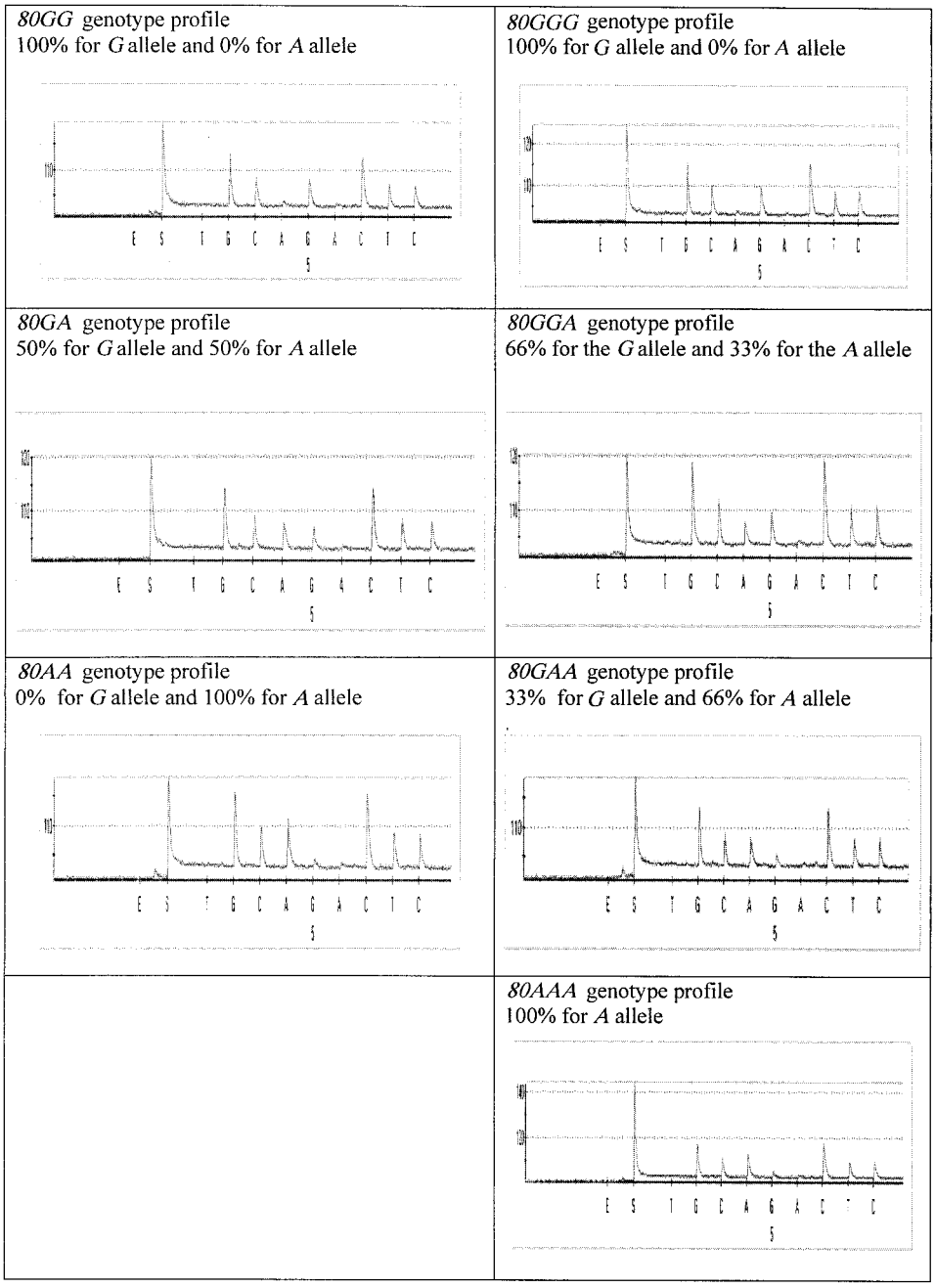

FIGURE 1. Quantification of the reduced folate carrier 80G→A single-nucleotide polymorphism (SNP) by pyrosequencing. The sequence to be analyzed was GGCA/GCCT. The sequencing primer was 5′-CTC CGG TCC TGG C. The SNP position was denoted A/G when entered in the PSQ 96MA software (Pyrosequencing AB, Uppsala, Sweden). The T and A at the first and sixth positions, respectively (**T**GCA/GA**C**CTC), were used as controls that should not bind to the DNA template and therefore should not result in a peak; the appearance of small peak heights at these positions is an indication of background in the assay. High peak heights at the G and C positions indicate the presence of the same adjacent nucleotide (GG or CC).

need specific techniques based on allele quantification. Pyrosequencing is an appropriate technique that allows one to distinguish heteroallelic individuals containing 1 or 2 copies of each allele.

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AC was the principal investigator and was responsible for developing the pyrosequencing technique and writing the manuscript. NFE contributed to collecting samples and analyzing the laboratory data. HB was the initial protocol designer responsible for collecting and managing the patients and samples during the study. JPN was the co-principal investigator and is the head of the medical biochemistry research group. FW developed the Agrohealth research program in the ISAB. None of the authors had any conflicts of interest.

REFERENCES

1. Chango A, Emery-Fillon N, Potier de Courcy G, et al. A polymorphism (80G->A) in the reduced folate carrier gene and its associations with folate status and homocysteinemia. Mol Genet Metab 2000;70:310–5.
2. Fillon-Emery N, Chango A, Mircher C, et al. Homocysteine concentrations in adults with trisomy 21: effect of B vitamins and genetic polymorphisms. Am J Clin Nutr 2004;80:1551–7.
3. Botstein D, White RL, Skolnick M, Davis RW. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet 1980;32:314–31.
4. Rickert AM, Premstaller A, Gebhardt C, Oefner PJ. Gentyping of SNPs in a polyploidy genome by pyrosequencing. BioTechniques 2002;32:592–603.

Erratum

Domellöf M, Lönnerdal B, Dewey KG, Cohen RJ, Hernell O. Iron, zinc, and copper concentrations in breast milk are independent of maternal mineral status. Am J Clin Nutr 2004;79:111–5.

On page 111, the last sentence of the abstract should read as follows: Milk iron concentrations increase and milk zinc concentrations decrease during weaning.