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Iron deficiency is uncommon among lactating women in urban Nepal, despite high risk of inadequate dietary iron intake

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ABSTRACT

The main objective was to describe dietary iron intake and dietary predictors of iron status and hemoglobin concentration among lactating women in Bhaktapur, Nepal. We included 500 randomly selected lactating women in a cross sectional survey. Dietary information was obtained through three interactive 24-h recall interviews including personal recipes. Hemoglobin (Hb) and the plasma concentration of plasma ferritin and soluble transferrin receptors (TfR) were measured. The daily median iron intake from food was 17.5 mg and 70% of the women were at risk of inadequate iron intakes. Around 90% of women had taken iron supplements in pregnancy. The prevalence of anemia was 20% (Hb<12.3 g/dL) and the prevalence of iron deficiency was 5% (plasma ferritin <15 µg/L). In multiple regression analyses there was a weak positive association between dietary iron intake and body iron [β (95% CI) 0.03 (0.014, 0.045)]. Among women with children <6 months, but not those with older infants, intake of iron supplements in pregnancy for at least 6 months was positively associated with body iron (P for interaction <0.01). Due to a relatively high dietary intake of non-haem iron combined with low bioavailability, a high proportion of the women in this study were at risk of inadequate intake of iron. The low prevalence of anemia and iron deficiency may be explained by the majority of the women consuming iron supplements in pregnancy.

INTRODUCTION

Iron deficiency is the most prevalent micronutrient deficiency globally. It is an important cause of anemia [1] which affects 42% of pregnant women and nearly a third of all non-pregnant women of reproductive age [2]. Low dietary iron content, often combined with low bioavailability from plant-based diets are important causes of iron deficiency, especially in pregnant women who have increased iron requirements[3].

The degree of iron absorption depends on the form of iron consumed and by the presence of dietary enhancers or inhibitors of absorption. Highly bioavailable haem iron is likely to be consumed infrequently and in small amounts by people in resource-poor settings. The majority of dietary iron in such countries is non-haem , which is often poorly absorbed due to the presence of dietary inhibitors such as phytate and tannins.

The present study was carried out in Bhaktapur municipality in semi-urban communities in the Kathmandu valley. A previous survey in this population reported an anemia prevalence of 12% and that 54% consumed inadequate amounts of iron[4]. The Demographic Health Survey (DHS) from 2011 showed that 39% of the lactating women in Nepal (15-49 years old) were anemic [5]. Another study among pregnant women in the southeastern plains of Nepal, reported that dietary haem iron intake was significantly associated with lower risk for iron deficiency without anemia[6]. However, few studies in developing country settings have related measures of dietary intake to biochemical measures of iron status [7]. Furthermore, a systematic review on dietary micronutrient intakes of women in resource poor settings concluded that there is a need for more documentation of the risk of inadequate micronutrient intakes among women living in low-income settings[8].

The main objective of this paper is to describe the dietary iron intake and dietary predictors of iron status and hemoglobin concentration in a representative sample among lactating women in Bhaktapur, Nepal.

METHODS

Study Area and Population

A cross-sectional survey was carried out among 500 randomly selected healthy lactating women (17-44 years old) from Bhaktapur municipality, Nepal. Bhaktapur is an urban area located 15 km east of the capital Kathmandu and was chosen because of the socio-economic diversity of this

population which gave us a unique opportunity to explore dietary variation. It has a total population of approximately 75,000, predominantly of the Newari ethnic group, and mostly farmers, semi-skilled or unskilled laborers and daily wage earners.

Study design and participants

From a public health perspective, we wanted to detect deficiencies of micronutrients such as zinc, iron and vitamin B12 with a prevalence of >25%. It was calculated that four hundred and fifty mother would be adequate to detect this prevalence with an absolute precision of 4% i.e. with a 95% confidence interval from 21%-29%. Assuming incomplete sampling from approximately 10% of these women we calculated a final desired sample size of 500 women. In the first stage of sampling we used a population proportionate to size method to select 66 of 160 geographic areas (*toles*). In the second stage, we obtained census lists of all women living in the 66 toles and selected randomly from these lists. We had to approach 582 women in order to enrol 500 women in the study (Figure 1). A total of 500 lactating (encompassing both exclusive and partial) women were enrolled in the study and completed the first 24-hour dietary recall. Due to dropouts, the sample sizes for the second and third 24-h recalls were 487 and 477, respectively. Eleven women were excluded due to errors in the interactive 24-h recalls and the final sample size consisted of 466 lactating women who had completed three 24-h recalls.

Women came to the hospital to receive physical examinations, dietary interviews, and blood draws. The first woman was enrolled in January 2008, and the last in February 2009. The inclusion criteria were that they were lactating, had no self-reported on-going infections, and were able to provide household information. Women with anemia (Hb<12.3 g/dL) were offered free treatment with iron supplements according to the national guidelines. All women gave written informed consent before start of the study which was approved by the ethical review board of the Institute of Medicine, Tribhuvan University. Women were classified as literate if they could read and write, and illiterate if they could do neither or only one of the two. Schooling was defined as: Primary school (1^{st} -3^{rd} grade); Secondary school (4^{th} -10^{th} grade); School Leaving Certificate, Intermediate school, or Bachelor degree.

Dietary Assessment

Nepali speaking, trained fieldworkers performed the interactive 24-h recalls and each woman participated in three interactive 24-h recalls[9] . Every fieldworker in this survey received training by a dietician for a period of two months. The fieldworkers were trained in interview techniques, how to use the electronic scales, how to estimate the volume of different foods, how to collect recipes, how to use the food codes, how to handle difficult situations and how to calibrate the weights before every interview. Fieldworkers were trained as a group but also practiced 24-h recalls on each other and on at least five women from the community prior to conducting recalls for the study. To ensure that the days represented normal intake and to minimize interviewer biases, recalls were obtained representing three different weekdays with 2-11 days separating each recall period and by three different fieldworkers. Saturdays (weekends) were excluded. The 24-h recalls were collected during one year covering the seasonal variation in food supply across participants in the study area. The same procedure was used to collect 24-h recalls for each of the three days: First, the women were asked to name all the food and drinks consumed during the preceding day, including anything consumed outside the home and time of consumption. Second, they were asked to describe ingredients and cooking methods for each recipe. Third, amounts of the foods and dishes were estimated using an electronic scale (Philips scales) with a precision of 1 g and a maximum capacity of 5 kg. The scales were calibrated daily. Cooked rice was used for estimating the volume of rice, vegetable stew (tarkari) and pickles and water was used to estimate the volume of lentils (*dal*). Fresh vegetables were used to measure the size and quantity of vegetables in the recipes. The amounts of meat, fish, bread and fruits were estimated by food models and pictures made exclusively for this study according to Gibson[9] . Clay models were used for estimating the portions of meat and fish, wooden models were used for bread whereas pictures were used for estimating the amounts of fruits consumed. Finally, the women were asked to recall snacks (foods consumed between meals) during the last 24 hours from a list of snacks, made especially for this study. The women's personal recipes were collected. Standard recipes were made for tea, spices (masala), lentils, bread, vegetable stew (tarkari) and pickle and were used when the women had bought ready-made food or when the food had been eaten at someone else's place. The standard recipes were developed from the collection of recipes in a pilot study and the average of the ingredients from at least 12 recipes for each dish was calculated. Information on consumption of fortified foods was not collected.

Most of the fortified food available in the area were designed for infants and preschool children and were thus not commonly consumed by adults.

Iron supplements in pregnancy

Information on consumption of iron supplements in pregnancy were collected through questionnaires. The women were encouraged at the hospital to take iron supplements during pregnancy and bought iron supplements from the hospital or in local drug stores. In the questionnaire the women were asked for how many months they consumed iron supplements in pregnancy and in what trimester they started. Information on consumption frequency, dosage or brand name were not collected, and henceforth iron supplement intake was not included in the analysis of iron intake. The common practice for provision of iron supplements was 60 mg of elemental iron sulfate from second trimester of pregnancy.

Nutrient Analysis

Because there is no standard Food Composition Table (FCT) available for Nepal, a FCT was compiled for this study "Composite Bhaktapur food composition table". In this FCT, nutrient values and foods were borrowed from World Food 2 (WF) [10], the "Nutritive Value of Indian Foods" [11]; and, where necessary, from Thai FCT [12] or US FCT [13]. The three 24-h dietary recalls and the "Composite Bhaktapur food composition table" were entered in nutrient analysis software designed exclusively for this study. The source of phytate data was the Indian Food Composition Table [11]. Intake of haem iron was calculated based on the assumption that haem iron makes up 40% of the iron in meat, poultry or fish. The usual intake distributions were calculated by the Multiple Source Method (MSM), which is characterized by a two-part shrinkage technique applied to residuals of two regression models, one for the positive daily intake data and one for the event of consumption [14, 15].

Estimation of adequacy of dietary iron intake

Estimation of dietary iron availability in this population was done according to Murphy et al [16], using quantitative data on intake of haem iron, non-haem iron, coffee and tea, as well as the amount of ascorbic acid and protein from meat, fish and poultry per 1000 kcal consumed. The average bioavailability of dietary iron intake in this population was calculated to be 4.5%. The

bioavailability of iron was also calculated according the algorithm developed by Bhargava and colleagues[17]. This algorithm includes estimation of iron stores, intake of fish and meat, ascorbic acid, phytate, non-haem - and haem iron, and is developed for Bangladeshi women, a population that might be equaled to our population. This algorithm gave an estimated average bioavailability of 1.8 %. WHO recommends use of calculation of 5 or 10% iron absorption in developing countries, depending on the diet [18]. We therefore used the 5% bioavailability assumption for the evaluation of the iron intake in this study.

The FAO/WHO's Estimated Average Requirements (EAR) and Recommended Nutrient Intake (RNI) 2004 were used to evaluate the nutritional adequacy of the women's diets depending on the time post-partum. The RNI for iron for lactating women (0-3 months) and women 19-50 years is 30mg and 58.8mg when the bioavailability is 5%. The risk of inadequate iron intake for women who had been lactating for less than three months was classified using the following definitions: Very high risk (average dietary iron intake below the EAR), moderate risk (average dietary iron intake between the EAR and the RNI), and low risk (average dietary iron intake higher than the RNI) [8]. A full probability approach was used [19], estimating the risk of inadequate iron intakes as a total product of the probability of inadequacy for a given range intake multiplied with the percentage of women with intakes in that range.

Anthropometric measurement

Measurements of weight of the women were conducted by using a UNICEF weighing scale (Salter, SECA, Germany). The height was measured with a locally made board in the clinic and calibrated weekly. Maternal body mass index (BMI) was calculated as kg/m^2. BMI<18.5 was considered underweight while 18.5 > BMI <25 was considered normal weight, and BMI ≥25 was considered overweight[20] .

Laboratory tests

During the first hospital visit, the first of the three 24 hours recalls was performed and a venous blood sample was collected from a cubital vein into a micronutrient-free, heparinized polypropylene tube (Sarstedt, Germany). The hemoglobin concentration was immediately measured by HemoCue (Vedbæk, Denmark) [21] which was regularly calibrated as recommended by the manufacturer. After centrifuging the sample at 760 x g for 10 min in room

temperature, plasma was transferred to micronutrient-free polypropylene vials (Eppendorf, Hinz, Germany) which were stored at -70 ^{o}C before transport on dry ice to Norway, and further stored at - 80 ^{o}C until analysis was performed at Laboratory of Clinical Biochemistry, Haukeland University Hospital, Bergen. The plasma concentration of the biochemical components was determined on a Modular Analytics System by Roche Diagnostics (Roche Diagnostics GmbH, Mannheim, Germany) with the analytical coefficient of variation (CV) being 5% for each test. Plasma ferritin was analysed by an electrochemiluminescence immunoassay (ECLIA), while the soluble transferrin receptor (TfR) was analysed by immunoturbidimetry.

Cut-off limits for the analytical test

Adjusted for altitude, anemia in this population was defined as hemoglobin <12.3 g/dl [(22)]. Mild anemia was defined as hemoglobin between 10.3-12.2 g/dL, moderate as 7.3-10.2 g/dL, and severe as hemoglobin less than 7.3 g/dL [(22)]. Iron deficiency, expressed as depleted iron stores, was defined by plasma ferritin <15 µg/L[(1)] . Increased need of iron in erythropoietic bone marrow and peripheral tissues was defined as TfR >4.4 mg/L [(23)]. As increased concentration of C-reactive protein (CRP) is a sensitive marker of inflammation, women with CRP > 5 mg/L were excluded from the analyses where plasma ferritin was involved.

Calculation of body iron stores

Body iron assessed as surplus (positive value) or deficit (negative value) of iron in the tissues, was calculated by using the formula described by Cook et al [(24)]. The formula is derived from a close linear relationship which was found between the logarithm of the ratio of the concentration of soluble serum TfR and plasma ferritin (R/F ratio, µg/µg) and body iron expressed as mg iron / kg body weight, corrected for the absorption of dietary iron: mg iron per kg = - [log(R/F ratio) – 2,8229] / 0,1207. Since analysis of TfR refers to the in-house ELISA-assay developed by Flowers et al.[(25)], TfR results obtained by the Roche method were converted by using the regression equation presented by Pfeiffer et al [(26)]:

*Flowers-TfR = 1.5**TfR-Roche + 0.35.*

Data processing and statistical analysis

Data were analyzed using SPSS version 17 (SPSS Inc., Chicago, IL, USA), STATA version 12 (StataCorp., College Station,TX, USA), and R version 2.16 (r-project.org). Continuous data that were not normally distributed were presented as median and 25 (P_{25}) and 75 (P_{75}) percentile. Two-tailed tests with a significance level of 5% were used for all analyses. We measured the association of relevant independent variables with the dependent variables (hemoglobin andbody iron) in multiple linear regression models. Variables that were known to influence hemoglobin and body iron as well as selected socioeconomic variables were included in the initial crude models. Candidate variables included dietary iron intake, vitamin C intake, vitamin A intake (beta carotene), phytate intake, use of iron supplements in pregnancy (for at least 6 months), mothers age, mothers BMI, mothers literacy, parity and the infant age at the time of the baseline visit. All covariates showing linear association (P<0.10) in the crude regression models were included in a preliminary multiple regression model. Variables that were still significantly associated in this model (P<0.10) were retained in the final model [(27)]. Analysis of the residuals was performed in order to examine the fit of the model. In the final model the following interactions between the independent variables were assessed and included if the interaction term was significant (P<0.10): (dietary iron intake x vitamin C (dichotomous variable, intake P_{25} (42 mg/d) and P_{75} (72 mg /d)) and (time since birth (dichotomous variable, cut-off 6 months) x prenatal iron supplement for at least six months). We explored and depicted the linearity of the associations between the independent and dependent variables in generalized additive models (GAM) [(28)]. We adjusted for clustering of outcomes due to the sampling design using the SVY group of commands for complex survey data in STATA.

RESULTS

The mean age of the 500 enrolled women was 25.8 years, and the majority were literate, had a healthy BMI and less than three children. Iron supplements in pregnancy. was reported by 90% of the women, for which the mean duration was 4.8 months and more than half initiated supplementation during the second trimester (Table 1).

Dietary intake of iron and risk of inadequate intake of iron

The dietary intake of iron and other dietary factors with the potential to affect iron absorption are presented in Table 2. The median daily iron intake from food was 17.5 mg and nearly all iron consumed was non-haem form (98%). The daily dietary intake of phytate (3304 mg/d) was well above the level known to adversely affect iron absorption and the main sources were rice, dal, potato and whole wheat flour. Enhancers of iron absorption (meat, fish and poultry; and vitamin C) were consumed in moderate amounts. The principal sources of dietary iron in this population were mustard leaves, rice flakes ("beaten rice"), turnip leaves, rice, and whole wheat flour (Table 3). Based on the WHO/FAO EAR for women consuming a diet with a low bioavailability (5%), 72.7% of the 33 women who were ≤3 month's post-partum were considered to have a very high risk of inadequate iron intakes. Only 15% of women ≤3 month's post-partum were at low risk of inadequate dietary iron intakes (Table 4). Using a full probability approach for women >3 months post-partum (n=432), the total prevalence of inadequate intake of iron was estimated to be 78%.

Iron Status and Anemia

Adjusted for the altitude of our study area (1400 m), the prevalence of anemia (Hb <12.3 g/dL) was 20% and most (17%) was mild anemia (Hb 10.3-12.2 g/dL). Only 5% of the women (n=26) had depleted iron stores with plasma ferritin < 15µg/L, while 15% (n= 73) had TfR > 4.4 mg/L indicating insufficient supply of iron to erythropoietic bone marrow and peripheral tissues. plasma ferritinOf those with plasma ferritin < 15 µg/l, 69 % (n=18) also had TfR >4.4 mg/L as a sign of empty iron stores with accompanying iron deficient erythropoesis evidenced by a mean hemoglobin concentration of 11.2 g/dL (SD 1,3). Thus, the prevalence in the whole study group of true iron deficient anemia, was 3.6 %. The remaining eight subjects with plasma ferritin < 15 µg/L and no increase in TfR, had sufficient supply of iron to the tissues despite depleted iron stores.

103 women had plasma ferritin between 15 and 35 µg/L, which is compatible with depleted or very small iron stores in many women[29]. In this subgroup 21 women had TfR > 4.4 mg/L indicating restricted hemoglobin synthesis due to insufficient supply of iron to the bone marrow. Since low plasma ferritin may be evidence of a negative iron balance, it is conceivable that 53 % (n=39 with plasma ferritin < 35 mg/L) of the 73 women with increased TfR-values, could be explained by restricted iron supply to the tissues.

Body iron calculated from the logarithm of the geometric mean of the R/F ratios (95.6), was 7.0 mg iron / kg body weight (SD 3,3) with a range between minus 8,9 to 14,5 mg iron/kg. 15 of the women (3 %) had negative values indicating tissue iron deficiency, with a mean of minus 2,8 mg iron / kg (SD 3,4).The rest of the group (485, i.e.97 %) had a mean body iron of 7,3 mg iron / kg (SD 5,5) (Table 5).

Dietary predictors of iron status and anemia

In the linear regression models (Table 6), intake of iron supplements in pregnancy predicted hemoglobin concentration. Dietary iron intake and iron supplements in pregnancy predicted body iron. Intake of iron supplements in pregnancy was associated with a 0.29 (95% CI: 0.04-0.54) g/dL higher hemoglobin concentration (P = 0.03). Dietary iron intake and potential enhancers and inhibitors of absorption were not associated with hemoglobin concentration. There was a weak positive association between dietary intake of iron and body iron 0.03 (95% CI: 0.014, 0.045). In addition, for women with children < 6 months, but not in those with older infants (P-for-interaction < 0.01), intake of iron supplements in pregnancy were positively associated with body iron. We identified the same predictors in the multiple linear regression models using ferritin concentrations and transferrin receptor as the dependent variables. R^2 in the models with ferritin and transferrin receptors were 0.22 and 0.09, respectively. The association of dietary iron intake with biochemical markers is also depicted in graphs from GAMs (Figure 2).

DISCUSSION

In this population of lactating women in Bhaktapur, we found that more than 98% of dietary iron intake was non-haem iron and the intake of phytate was high. Therefore, >70% of these women were estimated to be at risk of inadequate intakes. At the same time only 5% had plasma ferritin indicative of iron deficiency and 15% of women had elevated TfR mainly indicative of iron deficient erythropoiesis. We demonstrated that dietary iron intake and iron supplements in pregnancy predicted body iron. Further, intake of iron supplements in pregnancy, but not dietary variables showed a strong positive association with hemoglobin concentration.

Dietary iron intake

The iron consumed by these Nepali women was almost exclusively non-haem iron. It should be noted that no fortified food staples were available to this population at the time of data collection. The main contributors of dietary iron intake were cooked green leaf relishes, unrefined whole meal bread *roti* and rice flakes; all of which contained significant amounts of phytate based on available food composition data [10, 11], and therefore impaired the bioavailability of iron. In addition, meat which enhances the uptake of non-haem iron [30] , was not commonly consumed. On the other hand, vitamin C intakes were about 70% of the RNI for lactating women and obtained from vegetable relishes commonly eaten with meals and may have had a positive impact on iron bioavailability. Tea consumption is unlikely to have had any substantially negative impact on iron bioavailability because it is typically not consumed with meals.

The dietary iron sources were similar to that of a previous study among non-pregnant, non-lactating women in Bhaktapur, but that study reported lower mean iron intakes (8.4 mg/d) [4]. This may in part be due to the different food composition tables used. The previous study used exclusively data from the World Food Dietary Assessment System [10] whereas we used primarily Indian food composition data [11] because Indian foods were more specific to food items consumed in Nepal. The iron content of mustard leaves, turnip leaves, rice flakes, refined rice and whole wheat flour was consistently higher in Indian food composition table, compared with similar substitute foods in World Food. These values may be higher than the true iron

content of these common Nepali foods. However, a study in the Kathmandu valley found that dietary iron intakes based on chemical analysis of 24-hour diet composites were three times higher than the calculated dietary intakes for the same 24 hour period using the USDA database [31]. Therefore it is possible that the iron content of the foods consumed in Bhaktapur is higher than estimated. There is a clear need for a Nepal-specific food composition table.

Probability of inadequacy

Based on plasma ferritin and TfR, we found the prevalence of iron deficiency to be 5.3% and 14.6 % respectively. Yet, we estimated that 70% of women were at risk of inadequate dietary iron intakes. There are several possible reasons for this discrepancy. First, by using a cut-off value of plasma ferritin < 15 µg /L for depleted iron stores, we have probably underestimated the prevalence of iron deficiency as signaled by this biomarker (the prevalence of iron deficiency was 25.8 % by using cut-off values for plasma ferritin < 35 µg /L). As shown in the study by Hallberg et al [29]., at this cut-off value the diagnostic sensitivity and specificity of plasma ferritin is 75% and 98%, respectively. They found that the iron stores could be negligible at plasma ferritin concentrations from 35 µg/L and downwards. Second, 90% of women had used iron supplements in pregnancy and more than half initiated supplementation in second trimester. Thus temporary iron supplementation in pregnancy is likely to have had a positive effect on the iron stores during the first few months of lactation, but probably not later [5] when iron loss increased due to the return of menstruation and probably because iron supplementation stopped. Third, we may have overestimated the probability of inadequate iron intakes because we used the EAR for non-pregnant non-lactating women for all who were >3 months post-partum. Many of these women may still have had lactation amenorrhea and therefore had lower iron requirements than what was the basis of the EAR used. However, the risk of inadequacy for women ≤3 months post-partum, for whom lactation amenorrhea was taken into account, was similar to those who had given birth earlier. Fourth, as discussed above, the food composition data used may not have accurately reflected the true iron, phytate and vitamin C content of Nepali foods. It is possible that the iron content of several foods is higher and/or the phytate content is lower than what estimated based on available data.

The calculation of body iron based on the ratio between the TfR and plasma ferritin concentration, gives a more precise picture of the tissue iron content than what is achieved by

using cut off limits of the biochemical tests. Our results in iron replete and iron deficient women were somewhat different from what was reported by Cook JD et al [24] in their study of women between 20 and 45 years of age. In women with normal iron status they found mean iron stores of 5.5 mg iron / kg (SD 3.35) versus 7.3 mg iron / kg SD 5.5) in our study, and in women with iron deficiency, they found a mean deficit in tissue iron of - 3,9 mg/kg (SD 3.23) versus – 2.8 mg iron / kg (SD 3.4) in our study. As discussed above, the iron status of our women may have benefited from iron supplementation and less iron loss due to amenorrhea.

Dietary predictors of iron status and anemia

We found a significant albeit weak positive association between body iron and dietary iron intake, and also demonstrated that this association was linear. The lack of association between dietary iron intake and hemoglobin concentration may in part be due to that only a small proportion of anemia in this population was IDA; 82% and 63% of the anemic women did not have low plasma ferritin or elevated TfR respectively. However, the GAM curve revealed a linear association between dietary iron intake and hemoglobin concentration at lower dietary iron intakes (Figure 2). This effect however is small compared to the strong association between intake of iron supplements in pregnancy and hemoglobin concentration in our study. As discussed earlier, this effect persists into the lactation period. In addition the 2011 NDHS showed that 80% of Nepali women took iron supplementation during last pregnancy and 41% consumed iron supplements post-partum [5]. Lastly, other micronutrient deficiencies may also have been responsible for lower hemoglobin concentrations in our study, such as folate, vitamin B-12 and vitamin A [32], but their relationships to hemoglobin were not studied.

Strengths and weaknesses

This study had a number of strengths. We had a representative sample of lactating women, with a relatively large sample compared with most other dietary studies. We had three interactive 24-h recalls with personal recipes adapted to local foods. We trained local staff to perform the recall interviews and the interviews were done throughout the year, covering the seasonal variation in food intake at a group level. Using plasma ferritin and TfR as biomarkers, the near linear association of their relevant changes with dietary iron intake indicates strong relative validity of our adapted interactive 24-h recall method. Inflammation will obscure the interpretation of

plasma ferritin, but not TfR which can explain the difference in prevalence of iron deficiency based on plasma ferritin and TfR. However, chronic and acute illness was an exclusion criteria in this study and very few (24 women) had elevated CRP. Therefore we believe that the difference in prevalence is not due to inflammation. The primary weakness of our study was the reliance of external food composition tables, which may not reflect the true nutrient composition of local Nepali foods. Our findings may have also been adversely affected by bias caused by the fact that participating women knew they were coming in for a dietary interview and may have altered their diet. However, the fact that few women had insufficient iron intake may suggest that potential bias would have been towards the overestimation, rather than underestimation of iron intake.

Conclusion

Probably due to a high dietary intake of non-haem iron combined with low bioavailability, a high proportion of the lactating women in this study were at risk of inadequate intake of iron. The low prevalence of anemia and iron deficiency may be explained by the majority of the women consuming iron supplements in pregnancy.

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Conflict of interest

Author disclosure: Henjum, Manger, Skeie, Ulak, Thorne-Lyman, Chandyo, Shresthsa, Locks, Ulvik, Fawzi, Strand have no conflict of interest.

Authorship

T.A:S., R.C., S.H., F.W. and S. P., designed the research; R. C., M. U., E. S., S. H. conducted the research; S.H., A. T-L., R.U., L.L., and T.A.S analyzed the data; S.H. and M.M. wrote the paper;

433 and S.H. and T. A. S. had primary responsibility for the final content. All authors read and
434 approved the final manuscript.

References

1. World Health Organization, United Nations Children's Fund, United Nations University. Iron Deficiency Anaemia Assessment, Prevention, and Control. A guide for programme managers. Geneva: WHO; 2001.
2. McLean E, Cogswell M, Egli I, *et al.* Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. Public Health Nutr. 2009;12:444-54.
3. Zimmermann MB, Hurrell RF. Nutritional iron deficiency. Lancet. 2007;370:511-20.
4. Chandyo RK, Strand TA, Ulvik RJ, *et al.* Prevalence of iron deficiency and anemia among healthy women of reproductive age in Bhaktapur, Nepal. Eur J Clin Nutr. 2007;61:262-9.
5. NDHS: Ministry of Health and Population (Nepal). Nepal Demographic Health Survey - 2011, Kathmandu, Nepal. New ERA and ICF International Inc. 2012 Macro. Calverton, Maryland, USA. In.; 2011.
6. Makhoul Z, Taren D, Duncan B, *et al.* Risk factors associated with anemia, iron deficiency and iron deficiency anemia in rural Nepali pregnant women. Southeast Asian J Trop Med Public Health. 2012;43:735-46.
7. Backstrand JR, Allen LH, Black AK, *et al.* Diet and iron status of nonpregnant women in rural Central Mexico. Am J Clin Nutr. 2002;76:156-64.
8. Torheim LE, Ferguson EL, Penrose K, *et al.* Women in resource-poor settings are at risk of inadequate intakes of multiple micronutrients. Journal of Nutrition. 2010;140:2051S-8S.
9. Gibson RS, Ferguson EL. An interactive 24-hour recall for assesing the adequacy of iron and zink intakes in developing countries: HarvestPlus; 2008.
10. Food and Agriculture Organization of the United Nations. Food Composition: Overview of the WorldFood Dietary Assessment System. 2010 [cited; Available from: http://typo3.fao.org/fileadmin/templates/food_composition/documents/World_food_dietary_assessment_system/Overview_of_the_WorldFood_Dietary_Assessment_System.pdf
11. Gopalan C, Rama Sastri B, Balasubramanian S. Nutritive Value of Indian Foods: National Institute of Nutrition; 1989.
12. Institute of Nutrition Mahidol University. Food Composition Database for INMUCAL Program: Thailand: Institute of Nutrition; 2002.
13. United States Department of Agriculture, Agricultural Research Service. Search the USDA National Nutrient Database for Standard Reference. 2012 [cited; Available from: http://ndb.nal.usda.gov/
14. Haubrock J, Nothlings U, Volatier JL, *et al.* Estimating usual food intake distributions by using the multiple source method in the EPIC-Potsdam Calibration Study. J Nutr. 2011;141:914-20.
15. Harttig U, Haubrock J, Knuppel S, *et al.* The MSM program: web-based statistics package for estimating usual dietary intake using the Multiple Source Method. Eur J Clin Nutr. 2011;65 Suppl 1:S87-91.
16. Murphy SP, Beaton GH, Calloway DH. Estimated mineral intakes of toddlers: predicted prevalence of inadequacy in village populations in Egypt, Kenya, and Mexico. Am J Clin Nutr. 1992;56:565-72.
17. Bhargava A, Bouis HE, Scrimshaw NS. Dietary intakes and socioeconomic factors are associated with the hemoglobin concentration of Bangladeshi women. Journal of Nutrition. 2001;131:758-64.
18. World Health Organization, Food and Agriculture Organization of the United Nations. Human Vitamin and Mineral Requirements; 2002.

19. World Health Organization, Food and Agriculture Organization of the United Nations. Guidelines for food fortification with micronutrients. In: Lindsay Allen, Bruno de Benois, Omar Dar, Richard Hurrell, editors. Geneva: World Health Organization,
Food and Agriculture Organization of the United Nations; 2006.
20. WHO. Global Database on Body Mass Index an interactive surveillance tool for monitoring nutrition transition; 2006.
21. Hemocue Corporate. Our Products - Hemoglobin Portfolio. 2011 [cited; Available from: http://www.hemocue.com/index.php?page=51.
22. CDC. CDC Criteria for anemia in children and child bearing-aged women. MMWR Morb Mortality Wkly Rep 1989. p. 400-4.
23. Kolbe-Busch S, Lotz J, Hafner G, *et al.* Multicenter evaluation of a fully mechanized soluble transferrin receptor assay on the Hitachi and cobas integra analyzers. the determination of reference ranges. Clin Chem Lab Med. 2002;40:529-36.
24. Cook JD, Flowers CH, Skikne BS. The quantitative assessment of body iron. Blood. 2003;101:3359-64.
25. Flowers CH, Skikne BS, Covell AM, *et al.* The clinical measurement of serum transferrin receptor. Journal of Laboratory & Clinical Medicine. 1989;114:368-77.
26. Pfeiffer CM, Cook JD, Mei Z, *et al.* Evaluation of an automated soluble transferrin receptor (sTfR) assay on the Roche Hitachi analyzer and its comparison to two ELISA assays. Clinica Chimica Acta. 2007;382:112-6.
27. Hosmer DW, Lemeshow S. Applied Logistic Regression. 2nd Edition ed. New York: John Wiley & Sons Inc; 2000.
28. Wood SN. Modelling and Smoothing Parameter Estimation with Multiple Quadratic Penalties. JRStatistSocB. 2000;62:413-28.
29. Hallberg L, Bengtsson C, Lapidus 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;85:787-98.
30. Bothwell TH, Charlton RW, Cook JD, *et al.* Iron metabolism in Man. Oxford, UK.: Blackwell Scientific Publications; 1979.
31. Moser PB, Reynolds RD, Acharya S, *et al.* 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.
32. WHO. Assessing the iron status of populations: a report of a joint World Health Organization/Centers for Disease Control technical consultation on the assessment of iron status at the population level. Geneva; 2004.
33. Suma RC, Sheetal G, Jyothi LA, *et al.* Influence of phytin phosphorous and dietary fibre on invitro iron and calcium bioavailability from rice flakes. Int J Food Sci Nutr. 2007:637-43.

FIGURE LEGENDS

FIGURE 1 Flow chart of the recruitment of study subjects.

FIGURE 2 Association between daily iron intake (mg) and hemoglobin (g/dL), plasma ferritin (μg/L) and plasma transferrin receptor (mg/L) in lactating women in Bhaktapur, Nepal (n = 466). The graph was made by using generalized additive models in R. The solid curves depict the estimated dose-response curve; the shaded areas represent the 95% CIs. The small vertical lines on the x axis show the distribution of the observations.

Table 1 Demographic and anthropometric characteristics of 500 lactating women in Bhaktapur, Nepal.

Women's demographic information	
Age, years[1]	25.8 (4.2)
Employed [2,3]	103 (21.0)
Years of school[1,4]	2.5 (1.0)
Literate[2]	379 (81.5)
Women's Body Mass Index (kg/m^2) [1,5]	22.5 (3.1)
< 18.5[2]	23 (4.6)
18.5-25[2]	366 (78.7)
> 25[2]	84 (17.0)
Parity[1]	1.9 (0.9)
One[2]	203 (41.0)
Two[2]	206 (41.0)
Three or more[2]	90 (18.0)
Iron supplements in pregnancy[2]	449 (89.8)
Months of iron supplementation (n=449) [1]	4.8 (1.9)
Started in first trimester[2]	144 (29.0)
Started in second trimester[2]	262 (52.4)
Started in third trimester[2]	43 (8.6)

[1] Mean (SD)

[2] Numbers (%)

[3] Work in agriculture, carpet factory, daily wage earner, self-employed, or services.

[4] Primary school (1st -3rd grade); Secondary school (4th -10th grade); School Leaving Certificate, Intermediate school, or Bachelor degree

[5] BMI < 18.5 was defined as underweight, BMI 18.5 - 25 was defined as normal weight and BMI > 25 was defined as overweight

Table 2 Dietary intakes (usual intake based on three 24-h recalls per women) of energy, nutrients, foods, and constituents influencing iron absorption in 466 lactating women in Bhaktapur, Nepal.

Variables	
Energy intake, kcal[1]	2024 (312)
Dietary iron, mg[2]	17.5 (13.3, 24.7)
Haem , mg[2]	0.3 (0.0, 0.6)
Available iron, mg[2,3]	0.8 (0.6, 1.3)
Vitamin C, mg[2]	55.8 (42.0, 71.7)
Calcium, mg[2]	461.0 (334.3, 632.4)
Meat, fish & poultry, g[2, 4]	54.0 (37.9, 72.5)
Protein meat, fish & poultry, g[2, 4]	18.35 (7.9, 23.7)
Dry tea, g[2, 5]	2.1 (1.5, 2.6)
Phytate, mg[1]	3304 (776)
Phytate:iron molar ratio[6]	15.9 (10.4, 24.3)

[1] Mean (SD)

[2] Median (25th, 75th percentile)

[3] 5% bioavailability calculated according to Murphy et al [(16)]

[4] (n=327)

[5] (n=425)

[6] Gibson and Ferguson[(9)]

Table 3 Main sources of dietary iron in Bhaktapur, Nepal.

Food	Iron contribution to intake (%)	Iron content (mg Fe/100g food)[1]
Mustard leaves	25.4	16.3
Rice flakes	50.0	20.0[2]
Turnip leaves	10.2	28.4
Rice	8.1	0.2
Wheat flour whole	4.9	4.9

[1] Value from the Indian FCT [(11)], [2] When using a value from Suma et al[(33)], the iron contribution to intake was 18% and the iron content 5.2 mg

Table 4 Proportion of different risk-groups of inadequate iron intake for 33 lactating women who ≤3 months post-partum consuming a diet with 5% iron bioavailability[1] in Bhaktapur, Nepal

Risk of inadequate iron intake	Dietary iron intake(mg)	Proportion[2]
Very high[3]	≤23.4	24 (72.7)
Moderate[4]	23.4 – 30.0	4 (12.1)
Low[5]	≥30.0	5 (15.2)

[1]Iron bioavailability calculated according to Murphy et al [16] and WHO recommendations [18]

[2] Numbers (%)

[3] Average dietary iron intake ≤EAR for lactating women

[4] Average dietary iron intake >EAR and ≤RNI for lactating women

[5] Average dietary iron intake >RNI for lactating women

Table 5 Haemoglobin, plasma ferritin and transferrin receptor among 500 lactating women in Bhaktapur, Nepal

	Mean (SD) or N (%) where indicated
Hemoglobin (g/dL)	13.1 (1.3)
Anemia < 12 g/dL	61 (12%)
Anemia <12.3 g/dL[1]	100 (20%)
Mild anemia10.3-12.2 g/dL[1]	87 (17.4)
Moderate anemia 7.3-10.2 g/dL[1]	13 (2.6%)
Severe anemia <7.3 d/dL[1]	0 (0%)
Plasma ferritin (µg/L) [2]	68.8 (46.2)
< 15 µg/L[2]	26 (5.3%)
< 35 µg/L[2]	129 (25.8%)
Plasma transferrin receptor (TfR), mg/L	3.4 (1.5)
> 4.4 mg/L[3]	73 (15%)
Ferritin < 15 µg/L and TfR > 4,4 mg/L	18 (3,6%)
C-reactive protein (CRP)	
>5.0 mg/L	24 (5%)
Body iron (mg iron/kg body weight)[4]	7,0 (3.3) (range: - 8,9 to 14,5)
Tissue iron deficit, N=15 (3%)	- 2,8 (3,4)
Storage iron present, N=485 (97%)	7,3 (5,5)

[1]Threshold for defining anemia is adjusted for altitude (22)

[2] (n=476), 24 women excluded due to elevated CRP

WHO cut-off for depletion of iron stores (21)

[3] Cut-off for iron deficient erythropoiesis deficiency (23)

[4] Negative value is a quantitative measure of tissue iron deficit (i.e. lack of stored iron)

Table 6 Multiple linear regression models of the relationship between dietary iron intake, hemoglobin concentration and body iron (n=500)

	Multiple adjusted[1] β (95% CI)	**P**	**Standardized beta coefficients**
Model 1 Hemoglobin (R^2 =0.02)			
Iron supplements in pregnancy [2,3]	0.29 (0.04, 0.54)	**0.03**	**0.11**
Model 2 Body iron, mg Fe/kg body weight (R^2 =0.20)			
Dietary iron (mg)	0.029 (0.014, 0.045)	**<0.01**	**0.16**
Time since birth x Iron in pregnancy[4]	2.69 (1.54, 3.84)	**<0.01**	
Iron supplements in pregnancy			
Time since birth < 6 months	2.72 (1.79, 3.65)	**<0.01**	**0.40**
Time since birth ≥ 6 months	0.03 (-0.67, 0.78)	**0.93**	**0.00**
Time since birth < 6 months			
Iron supplements in pregnancy	2.24 (1.36, 3.13)	**<0.01**	**0.33**
Iron not supplements in pregnancy	-0.45 (-0.29, 1.18)	**0.23**	**-0.07**
Mothers age[5]	0.21 (0.14, 0.28)	**<0.01**	**0.26**
Literacy[6]	0.81 (0.25, 1.39)	**<0.01**	**0.12**
Ownership of land[6]	0.74 (0.19, 1.30)	**<0.01**	**0.11**

[1] Both models included mother's age, parity, literacy, and child's age

[2] Iron supplements in pregnancy at least 6 months, dichotomous variable (yes/no)

[3] No significant interaction between time since birth (dichotomous variables cut-off 6 months) and iron supplements in pregnancy at least 6 months

[4]Interaction between time since birth (dichotomous variables cut-off 6 months) and iron supplements in pregnancy at least 6 months

[5]Continuous variable

[6]Dichotomous variable (yes/no)