Erythrocyte Riboflavin for the Detection of Riboflavin Deficiency in Pregnant Nepali Women, Joanne M. Graham,† Janet M. Peerson,‡ Marjorie J. Haskell,‡ Ram K. Shrestha,§ Kenneth H. Brown,† and Lindsay H. Allen¶

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Riboflavin deficiency is common among chronic alcoholics, the elderly, and vegetarians (1–4), but intake in the United States is generally adequate (5,6), unlike the widespread deficiency in regions of the world with limited animal food sources (7,8). Riboflavin status has been assessed from measurements in urine, plasma, and erythrocytes (9–11). The erythrocyte glutathione reductase activity coefficient (EGRAC) is the commonly used test and reflects the adequacy of riboflavin to support enzyme function (10). In this assay, the stimulation of erythrocyte glutathione reductase by FAD is measured in vitro, and a higher activity coefficient reflects a larger amount of unsaturated glutathione reductase apo-enzyme resulting from lack of FAD. The EGRAC cutoffs (12) are based on observational studies in well-nourished American school children (n = 431) and adult men (n = 6) and a depletion–repletion study in Indian adults (n = 8) (13–15). These cutoffs are usually ≥1.4 for deficiency status, 1.2 to <1.4 for marginal status, and <1.2 for acceptable status.

Although erythrocyte riboflavin concentration is seldom used to assess riboflavin status, Hustad et al. (16) showed that the sum of erythrocyte flavin mononucleotide and FAD is correlated with the EGRAC and might be a useful indicator of riboflavin status in population studies. The elderly Irish persons (n = 122) in their study had marginal riboflavin status (mean EGRAC, 1.26), but no erythrocyte riboflavin cutoffs were evaluated. In healthy Californian adults (n = 22), we previously determined the 5th percentile of erythrocyte riboflavin concentration to be 170 nmol/L (range, 169–289 nmol/L) but did not measure the EGRAC in the same samples (L.H. Allen, unpublished data).

The purpose of our study was to compare erythrocyte riboflavin concentrations against the EGRAC in a group of pregnant women at risk for riboflavin deficiency. Because iron status can affect hemoglobin synthesis and erythrocyte production, the association of markers of riboflavin status with hemoglobin and plasma ferritin concentrations was compared. The erythrocyte riboflavin cutoff with the greatest sensitivity and specificity for detecting deficiency was determined by ROC analysis, with the EGRAC as the reference value.

The participants were women (n = 84) in their first to seventh month of pregnancy who had self-reported night blindness before participating in a large food-based vitamin A treatment trial (17). They were reportedly healthy and had no signs of xerophthalmia. The research was conducted from August to October 2000 in 52 village development communities of the Saptari District in southeastern Nepal. Approval was obtained from the ethics review committees at the University of California, Davis, and the Nepal Health Research Council, and informed consent was obtained from each participant.

Venous blood samples (7.5 mL) were collected into S-Monovette® (Sarstedt) tubes containing lithium heparin as anticoagulant. Hemoglobin was measured immediately by a portable hemoglobinometer (HemoCue®). Plasma was aliquoted into duplicate 1.5-mL cryovials. Erythrocytes were processed under dim light by washing 3 times with cold saline (9 g/L NaCl) followed by centrifugation for 10 min at 1500g. Washed erythrocytes were stored at −20 °C in duplicate 1.5-mL amber-glass microcentrifuge tubes to minimize riboflavin degradation. Plasma ferritin was quantified by a 2-site immuno-radiometric assay (Coat-A-Count IRMA; Diagnostic Products Corporation). EGRAC and erythrocyte riboflavin measurements were conducted within 2 months of each measurement.
anterior. Pooled blood from local healthy volunteers served as external controls for both methods.

Erythrocyte glutathione reductase activity was measured in duplicate samples by an automated plate reader (Multiskan Ascent; MTX Lab Systems, Inc.) with a 340 nm filter (12, 18). After incubation at 37°C for 30 min, NADPH (15 μL) was added to all wells, and the plate was shaken for 30 s. Samples were read immediately at 340 nm \( t_0 \) and again after a 12-min incubation at 37°C \( t_{12} \). The ratio of the difference in absorbance values \( [(stimulated_0 - unstimulated_0) - (stimulated_{12} - unstimulated_{12})] \) was used to calculate the activation of erythrocyte glutathione reductase. Analyses were repeated if the CV was >10%, and a CV <15% after one rerun was considered acceptable.

Erythrocyte riboflavin (riboflavin and FAD) was quantified by HPLC (1100 Series system; Agilent Technologies, Inc.) after modification of a previously published method (19, 20). Riboflavin and FAD calibrators were prepared by dissolving 3.8 mg of riboflavin or 8.3 mg of FAD in 100 mL of doubly distilled H₂O. Calibrators were dispensed in 1.0-mL aliquots into 1.5-mL amber-glass microcentrifuge tubes and stored at -70°C. An acetonitrile–sodium phosphate (25:75 by volume) mobile phase was made by dissolving 12.0 g of NaH₂PO₄ to 1 L of double distilled H₂O and bringing the solution to pH 2.9 by slow titration with 4 mol/L H₃PO₄. The mobile phase solution was vacuum-filtered through 47-mm 0.2 μm nylon filters and completed by adding 250 mL of acetonitrile (HPLC grade; Sigma) to yield 1 L.

Processed erythrocytes were thawed, diluted (100 μL of sample to 400 μL of double distilled H₂O), deproteinized with 100 μL of 300 g/L trichloroacetic acid in acetonitrile, immediately vortex-mixed (5–10 s), and placed on ice for 5 min. After centrifugation (15 min at 20,000g and 4°C), the supernatant was aliquoted into amber-glass HPLC vials, capped tightly, and placed in a water bath at 85°C for 10 min to complete acid hydrolysis. The vials were placed in the autosampler rack while they returned to room temperature and then were analyzed by HPLC using a normal-phase 0.5μ NH₂ column (Luna®; Phenomenex) with fluorescence detection at an excitation wavelength of 446 nm and an emission wavelength of 526 nm. The injection volume was 100 μL, the flow rate was 1 mL/min, and the run time was 6 min. The calibrators, control, and samples were run in duplicate. Samples with CVs >10% were reanalyzed.

Riboflavin deficiency was defined as EGRAC ≥1.4 (12), anemia was defined as hemoglobin <110 g/L (21), and iron deficiency was defined as plasma ferritin <12 μg/L. Statistical analyses were performed with PC-SAS 8.1 (SAS Institute, Inc.). Erythrocyte riboflavin and plasma ferritin concentrations were normalized by log transformation. We used Pearson correlation and linear regression analyses to examine relationships between variables. In all models, the EGRAC was used as the independent variable, and erythrocyte riboflavin was used as the dependent variable. Hemoglobin, month of pregnancy, and ferritin were included as covariates in linear regression models to control for anemia, hemodilution, and iron status. We used ROC analysis to compare the performance of erythrocyte riboflavin for determining riboflavin deficiency with EGRAC (22, 23). P <0.05 was accepted as statistically significant.

Both assessment methods showed that most of the women were riboflavin deficient (Table 1), similar to the 47% to 84% of pregnant women reported in other developing countries (24–27). A large proportion (75%) of women with iron deficiency were also anemic.

The log erythrocyte riboflavin concentration was negatively correlated with EGRAC \( (r^2 = 0.390; P <0.0001; y = -0.4503x + 5.726 \text{ nmol/L}; \text{Fig. 1A}) \). Hemoglobin was positively associated with erythrocyte riboflavin \( (P <0.005) \). No other covariates were significantly associated with erythrocyte riboflavin or EGRAC. In multiple regression models, EGRAC \( (P <0.0001) \) and hemoglobin \( (P = 0.01) \) were significant predictors of erythrocyte riboflavin. On the other hand, erythrocyte riboflavin \( (P <0.0001) \) was the only significant predictor of EGRAC. This indicates that it is not necessary to measure hemoglobin when

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<th>Table 1. Profile of participants (n = 84) and multiple regression statistics for both riboflavin status indicators.</th>
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<tr>
<td><strong>Mean (SE)</strong></td>
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<td>Age, years</td>
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<tr>
<td>Month of pregnancy</td>
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<td>Weight, kg</td>
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<td>Height, cm</td>
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<td>Body mass index, kg/m²</td>
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<td>Plasma ferritin, μg/L</td>
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<td>Erythrocyte riboflavin, nmol/L</td>
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* a Predictor variables in the model were hemoglobin, ferritin, month of pregnancy, and the corresponding riboflavin test.
* b Percentage deficiency defined as <110 g/L for anemia, <12 μg/L for iron, and ≤1.4 (EGRAC) or <170 nmol/L (erythrocyte) for riboflavin.
* c For plasma ferritin and erythrocyte riboflavin, results are the geometric means (SE) with lower and upper 95% confidence limits.
using erythrocyte riboflavin to assess riboflavin status; however, when hemoglobin, ferritin, and month of pregnancy were included in the multiple regression models, the correlation coefficient for each indicator of riboflavin status increased.

We used ROC analysis to examine the validity of an erythrocyte riboflavin concentration of 170 nmol/L as a cutoff for riboflavin deficiency. The sensitivities (true-positive fractions) and specificities (true-negative fractions) of the test to determine deficiency based on EGRAC ≥1.4 were calculated and plotted (Fig. 1B). A cutoff of 170 nmol/L erythrocyte riboflavin detected 92% of those with increased EGRAC values and 73% of those with normal EGRAC values. Lowering the cutoff to 164 nmol/L optimized both the sensitivity and specificity at 82%; if failure to identify a case of deficiency might pose more harm than identifying and treating cases of nondeficiency, the loss of specificity at the higher cutoff could be acceptable.

The ability of erythrocyte riboflavin to detect deficiency was further confirmed by plotting the true-positive fraction against the false-negative fraction. We used ROC analysis to examine the validity of an erythrocyte riboflavin concentration of 170 nmol/L as a cutoff for riboflavin deficiency. The sensitivities (true-positive fractions) and specificities (true-negative fractions) of the test to determine deficiency based on EGRAC ≥1.4 were calculated and plotted (Fig. 1B). A cutoff of 170 nmol/L erythrocyte riboflavin detected 92% of those with increased EGRAC values and 73% of those with normal EGRAC values. Lowering the cutoff to 164 nmol/L optimized both the sensitivity and specificity at 82%; if failure to identify a case of deficiency might pose more harm than identifying and treating cases of nondeficiency, the loss of specificity at the higher cutoff could be acceptable.

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Nucleophosmin (NPM1) is a multifunctional, highly conserved protein found most frequently in nucleoli. NPM1 acts as a molecular chaperone (1) and is thought to participate in preribosome maturation and centrosome duplication (2); in addition, it has been implicated in the regulation of the ArF-p53 tumor suppressor pathway (3, 4).

NPM1 mutations have recently been reported to occur at high frequency in acute myeloid leukemia (AML), for which they currently represent the most common detectable genetic lesion (~35% of cases). This abnormality is strongly associated with normal-karyotype AML and has never been detected in AMLs bearing major cytogenetic abnormalities. It has not been observed in other hematopoietic tumors (5). Two main types of mutations have been described to date. The first and most frequent consists of a 4-nucleotide (nt) insertion (YW TG; YUPAC code) downstream from nucleotide 959; the second is deletion of a GGAGG sequence at positions 965 through 969 and substitution with 9 extra nt (GenBank accession no. NM_002520). Both mutations lead to aberrant cytoplasmic localization of NPM1 as shown after immunostaining with anti-NPM1 monoclonal antibodies. In addition to their high frequency and clustering with normal karyotype, NPM1 mutations may identify a subset of AMLs with distinct response to therapy (5). Together, these findings may have repercussions in AML classification and suggest that analysis of NPM1 mutational status should integrate modern genetic characterization of AML. We describe here a rapid and reproducible method for screening NPM1 mutations by reverse transcription-PCR followed by denaturing HPLC (DHPLC).

Bone marrow samples showing at least 70% bone marrow infiltration by leukemic cells were collected at diagnosis from 56 patients with newly diagnosed AML observed at the Department of Biopathology at the University Tor Vergata (Rome). According to the French–American–British classification (6, 7), the following subtypes were included in the study population: 6 M0, 9 M1, 14 M2, 15 M4, 6 M5a, 4 M5b, and 2 M6. Fifty-three of 56 patients were evaluable for karyotype. Of these, 10 patients had favorable karyotypes [5 with t(8;21) and 5 with inv(16)], 30 patients had intermediate karyotypes [20 with