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Biomarkers of vitamin A status: what do they mean?

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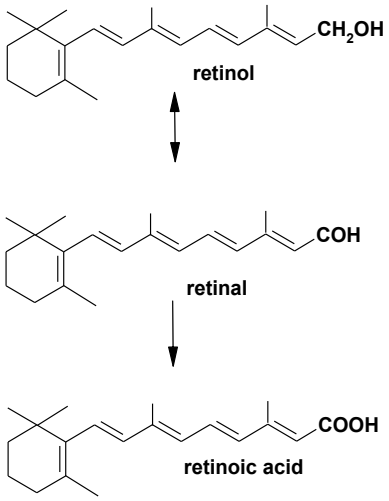
Abstract

Vitamin A is essential for growth, reproduction and immunity. Biomarkers of vitamin A status are diverse, in part, due to its functions. Liver reserves of vitamin A are considered the gold standard but this measure is not feasible for population evaluation. Biomarkers of status can be grouped into two categories: (1) biological, functional and histological indicators; and (2) biochemical indicators. Historically, signs of xerophthalmia were used to determine vitamin A deficiency. Before overt clinical damage to the eye, individuals with vitamin A deficiency are plagued by night blindness and longer vision restoration times. Surrogate biochemical measures of vitamin A status, as defined by liver reserves, have been developed. Serum retinol concentration is a common method used to evaluate vitamin A deficiency, but it is homeostatically controlled until liver reserves become dangerously low. Therefore, other biochemical methods that respond to liver reserves in the marginal category have been developed, such as dose response tests and isotope dilution assays. Dose response tests work on the principle that as liver reserves become depleted, apo-retinol-binding protein builds up in the liver. A challenge dose of vitamin A binds to this protein and serum concentrations increase within a few hours if liver vitamin A is low. Isotope dilution assays use stable isotopes to trace total body reserves of vitamin A. Different biomarkers have utility across a range of liver values.

Introduction

Vitamin A has a role in many functions including growth, vision, epithelial differentiation, immune function and reproduction (*1*). The storage form is retinol esterified to fatty acids, e.g. palmitic and oleic acids. Retinal is involved in vision and retinoic acid is involved in growth and cellular functions (**Figure A2.2.1**). According to the World Health Organization (WHO) (*2*), 45 countries have vitamin A deficiency of public health significance, which includes overt signs of deficiency, and 122 countries have subclinical levels of vitamin A depletion with marginal liver reserves. Many women and children have vitamin A deficiency that leads to vision loss and increased morbidity and mortality. While progress has been made globally to alleviate overt signs of vitamin A deficiency, marginal vitamin A status is still prevalent and difficult to diagnose.

Figure A2.2.1
Chemical structures of important functional forms of vitamin A: retinol is the major form in the circulation and is bound to fatty acids in the liver for storage until needed; retinal is involved in vision; and retinoic acid is involved in growth and cellular functions.



Due to concerns related to marginal vitamin A status, biomarkers have been developed to diagnose different degrees of vitamin A status. In 2010, these indicators were reviewed (*3*) and ranked against a continuum of liver reserves (**Figure A2.2.2**). Vitamin A biomarkers can be grouped into two categories: (1) biological, functional and histological indicators; and (2) qualitative and quantitative biochemical indicators. This brief review of these categories attempts to relate the indicators to predicted liver stores of vitamin A.

Figure A2.2.2
Biomarkers of vitamin A status in relation to liver reserve concentrations, which were proposed in 2010 at the Biomarkers of Nutrition for Development meeting with regard to the utility of isotope dilution testing in the hypervitaminotic state.

VITAMIN A (VA) STATUS CONTINUUM					
VA status	**Deficient**	**Marginal**	**Adequate**	**Sub-toxic**	**Toxic**
Liver VA	**<0.07**	**0.07–0.1**	**0.1–1.0**	**>1.0**	**10 µmol/g**
Indicator					
Clinical signs and tests					
Serum retinol					
Breast milk retinol					
Dose response tests					
Isotope dilution					
Liver sample					

Reproduced with permission from reference (*3*).

Review of indicators

Biological, functional and histological indicators

The first group of biological indicators is clinical and involves the eye. If an individual presents with ophthalmic signs of vitamin A deficiency, they need to be treated with high-dose supplements. Xerophthalmia has different degrees of severity ranging from Bitot's spots, which are reversible with vitamin A treatment, to irreversible blindness due to scarring of the cornea. Xerophthalmia is a population indicator and a minimum prevalence of Bitot's spots of 0.5% in preschool-age children is considered a public health problem (*4*).

Night blindness is a functional indicator and results when the vitamin A pool in the eye becomes depleted and the concentration in the rod cells is lowered. Many local languages have a specific term for this symptom of vitamin A deficiency. Night blindness due to vitamin A deficiency is reversible with increased vitamin A intake or supplementation. In countries where marginal vitamin A status is prevalent, night blindness may transiently occur during pregnancy. Whether this is due to increased demands during pregnancy or lowered serum retinol concentration due to an increase in plasma volume is not entirely known. Night blindness and impaired dark adaptation have been used to evaluate intervention studies (*5*, *6*). Specifically, dark adaptation measured by pupillary threshold in night-blind Nepali women improved when liver, fortified rice, amaranth leaves, carrots or retinyl palmitate were consumed for 6 weeks (*5*). If a population has a high prevalence of night blindness, the population should be considered to be at risk for vitamin A deficiency. This is not likely to occur until liver reserves are dangerously low, i.e. below the level considered to be deficient (0.07 μmol/g liver).

Qualitative biochemical indicators

Serum retinol concentration

Serum retinol concentrations are the most common population indicator. In addition to analysis with high-performance liquid chromatography (HPLC), surrogate analyses for the carrier protein retinol-binding protein (RBP) have been developed using either serum (*7*) or blood spots (*8*). The ratio of retinol to RBP may be influenced by vitamin A deficiency (*9*) or obesity (*10*), which may negatively affect prevalence rates of vitamin A deficiency when expressed as RBP concentrations. During deficiency, RBP accumulates in the liver and may be released unbound to retinol. In the case of obesity, adipose tissue synthesizes RBP that is released into circulation not bound to retinol. Both serum retinol and RBP concentrations are static measures and may not always change in response to an intervention. For example, in Indonesian children the initial and final serum retinol concentrations did not differ between groups that received 210 μmol vitamin A and those that did not 3–4 weeks after supplementation; the after to before ratio range was 0.96 to 1.03 (*11*).

On the other hand, serum retinol concentration distribution curves may have distinct differences between groups of children (*12*). When used as an evaluation tool, serum retinol distribution differed in children between two areas in Indonesia. However, in this study the degree of infection was not assessed. Therefore, the effect of correction for inflammatory markers on the distribution curves is not known (*13*). Infection and inflammation have a negative effect on serum retinol concentrations because RBP is an acute phase protein.

In women, serum retinol concentrations have responded to vitamin A supplementation if values are initially low, such as in Indonesian women given low-dose supplements for 35 days (*14*). However, in some groups, serum retinol concentrations may not respond even to high-dose supplements, such as in Ghanaian women who were given 210 or 420 μmol retinyl ester (*15*) or consumed indigenous green leafy vegetables for 3 months (*16*). The lack of response of serum retinol concentration is due in part to its homeostatic control over a wide range of

liver reserves. For example, in rats given three different levels of daily vitamin A supplements, serum retinol concentrations did not differ despite a sixfold difference in liver reserves of vitamin A (*17*).

Serum retinol is not a reflection of the vitamin A liver stores because it is homeostatically controlled and it does not drop until liver reserves are very low. The cut-off value for definition of deficiency has been discussed. In children certainly values <0.35 µmol/L and in women 0.70 µmol/L may indicate deficiency. However, if no infection is present in the population under study, 0.70 and 1.05 µmol/L for children and women, respectively, may be more descriptive of the actual status. If the population has access to a source of preformed vitamin A, serum retinol concentrations will be higher but not necessarily reflective of status. For example, in rats given a small daily dose of preformed retinol, serum retinol was normal at 1.37 ± 0.21 µmol/L even though liver reserves were extremely low at 0.005 µmol/g liver (*18*). The current widely accepted cut-off for deficient liver reserves is <0.07 µmol/g liver and a recent evaluation of animal data suggests that this should be raised to 0.1 µmol/g liver (discussed below).

As a population assessment tool, markers of inflammation should be used to adjust the serum retinol concentration. An analysis using sandwich enzyme-linked immunosorbent assays was able to quantify ferritin, transferrin receptor, RBP and C-reactive protein in a 30 µL serum sample (*19*). Considering the limitations of serum retinol as a reflector of status, an inexpensive assay for RBP linked with inflammation markers may be more practical even if it overestimates values because of the circulating unbound plasma RBP in individuals who are deficient.

Breast milk retinol concentration

Breast milk retinol concentration is a unique indicator in lactating women (*20*) with potential extrapolation to the nursing infant. Breast milk retinol concentrations can be used as an evaluation tool in groups of lactating women, although the response to supplementation was found to be modest in a sample of Kenyan mothers (*21*). As a biomarker, breast milk retinol concentrations may reflect recent dietary intake and not necessarily be a reflection of vitamin A status, as shown in rats (*22*) and swine (*23*). A comparison between vitamin A indicators suggests that casual breast milk retinol may perform better when corrected for fat content (*24*). Breast milk fat content and the fact that most retinol is esterified to fatty acids necessitate the use of saponification for analysis before HPLC. This requires special analytical considerations (*20*).

Quantitative biochemical indicators

Indirect semi-quantitative and quantitative methods include dose response and isotope dilution tests. Dose response tests have utility from deficiency through to the adequate range of vitamin A liver reserves. However, they probably do not quantitatively reflect status above the adequate range. Isotope dilution tests give a quantitative estimate of liver reserves from deficiency through to toxic vitamin A status (**Figure A2.2.2**).

Dose response tests

Dose response tests work on the principle that as vitamin A liver reserves become low, RBP accumulates. In rats fed a vitamin A-deficient diet, apo-RBP accumulated in the liver before serum retinol concentrations decreased and the liver was depleted (*25*). Thus, when a challenge dose of retinyl or 3, 4-didehydroretinyl ester is administered, the retinol or 3,4-didehydroretinol binds to this accumulated RBP and is rapidly released into the serum. The recommended dose for the relative dose response (RDR) test is 1 mg of retinyl ester dissolved in oil (*12*). Two blood samples are collected, i.e. the first one at baseline and another one 5 hours after dosing.

The RDR value, which is expressed in per cent, is calculated as follows:

$$[(A5 - A0)/A5]*100$$

Where: A5 is the serum retinol concentration at 5 hours post-dosing
A0 is the serum retinol concentration at baseline

If the per cent difference is >20%, the individual probably has deficient liver reserves <0.07 μmol/g liver.

While the RDR test is more descriptive than serum retinol concentrations alone, the test is somewhat invasive because it requires two blood samples from the same individual within a 5-hour interval. Furthermore, an accurate RDR value is dependent on correct analysis and consistent retinol recovery from both serum samples. Therefore, the modified relative dose response (MRDR) test was developed by Tanumihardjo et al. (*26–28*) and applied to humans (*28–30*). The test was refined by establishing standard doses of the test dose dependent on the age group and suggesting a range of response times to obtain the blood sample (*31*). The test works on the same principle as the RDR test, but because the 3, 4-didehydroretinol analogue is administered instead of retinol, a single blood sample can be taken. HPLC easily separates 3, 4-didehydroretinol from retinol in the same sample. Endogenous concentrations of 3, 4-didehydroretinol are low in humans and therefore a baseline blood sample is not needed. A distinguishing response between depleted and sufficient liver reserves can be measured in as little as 4 hours and has been validated in animals against liver vitamin A reserves (*26–28*, *32*). After the serum sample has been analysed, the 3, 4-didehydroretinol to retinol molar ratio is calculated, sometimes referred to as the MRDR value. A cut-off of 3, 4-didehydroretinol to retinol >0.060 usually suggests low liver reserves of retinol that can be corrected with vitamin A supplementation (*11*, *33*).

The MRDR test has been applied in several countries in order to evaluate population status, response to interventions and prevalence of low liver reserves in surveys. One of the first comparisons was done in two groups of Indonesian preschool-age children (*12*). In one group, the distribution of values approached a normal distribution, while the MRDR values were quite disparate with a value of 0.83 in one female subject. The application of the MRDR test in a study of combined treatment with vitamin A and albendazole for deworming children infected with *Ascaris lumbricoides* showed significant improvement in the mean 3, 4-didehydroretinol to retinol ratio of 0.055 ± 0.042 before supplementation to a ratio of 0.033 ± 0.017 after supplementation ($P < 0.0001$) (*11*). This magnitude of difference was not seen with serum retinol concentrations. In another evaluative study of children with helminthic infections, the MRDR test correctly identified children who had received a vitamin A supplement from the local health post (*33*). The MRDR values were 0.021 ± 0.012 and 0.054 ± 0.038 in the children who did and did not receive the supplement, respectively. Serum retinol concentrations did not differ between those who had received the supplement and those who had not, and nor did the serum retinol concentrations respond to treatment.

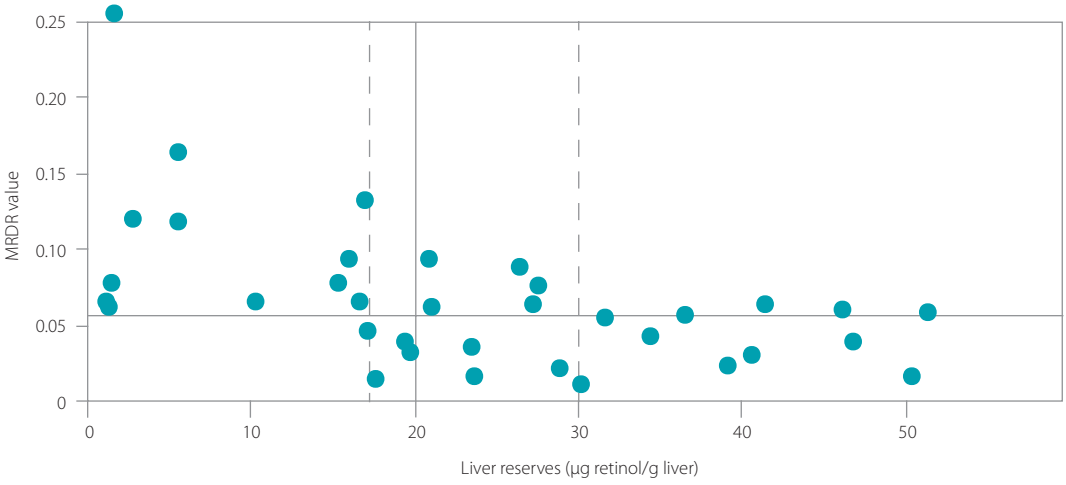
The MRDR test gives more information than serum retinol concentrations alone. For example, in a group of rural lactating women in Ghana, baseline serum retinol concentrations and MRDR values were 1.4 ± 0.5 μmol/L and 0.048 ± 0.037, respectively (*15*). After treatment with either 210 or 420 μmol retinyl ester, a significant improvement in vitamin A status occurred as assessed by the MRDR test ($P < 0.0001$), but serum retinol concentrations did not differ ($P = 0.87$). Furthermore, in an urban group of Ghanaian lactating women, the baseline serum retinol concentration was 1.5 ± 0.6 μmol/L and the MRDR value was 0.09 ± 0.05 (*16*), indicating a much poorer vitamin A status in these women compared with the rural women even though the serum retinol concentrations were identical. After use of an intervention with indigenous African green leaves, serum retinol concentrations did not change or differ during the study

($P > 0.41$), but the MRDR test improved within the intervention group ($P = 0.0001$).

In the USA, vitamin A status can be poor, especially among low-income groups. Specifically, in children qualifying for the Special Supplemental Nutrition Program for Women, Infants, and Children, 32% were in the uncertain area for MRDR values, which is defined as 0.030–0.060 (34). This is in contrast to children from a generally higher economic status in the USA, where the mean MRDR value was 0.019 ± 0.010 in 22 children tested 2–10 hours after an oral dose of 3, 4-didehydroretinyl acetate (*29*). Only two children tested >0.030 at 4 and 6 hours after the dose, which is within the recommended time interval for the test sample to be taken (*31*). Furthermore, an assessment of low-income pregnant women showed that an alarming 9% were above the international MRDR cut-off of 0.060 (*35*). Serum carotenoid concentrations were analysed in these low-income women and children and in some cases β-carotene was not detectable, indicating that vegetable consumption was likely very low (*34*, *35*).

Although the MRDR test is very useful in evaluating a deficient through normal vitamin A status, as currently applied, it does not have utility in defining the sub-toxic and toxic range of liver reserves. However, the magnitude of the ratio is related to liver reserves. When data from several piglet studies were combined (*32*, *36–38*), liver reserves <17 µg/g liver (0.06 µmol/g liver) were exclusively associated with an MRDR value of 0.060 (**Figure A2.2.3**). Liver values from 0.06 to 0.1 µmol/g liver were scattered above and below the cut-off and liver values >0.1 µmol/g liver were almost invariably associated with values <0.060. From these data and those obtained in rats where down-regulation of lecithin:retinol acyltransferase, which is responsible for retinol esterification, occurred at the same liver concentration (*39*), the author proposes that 0.1 µmol/g liver be used to define vitamin A deficiency instead of the current cut-off of 0.07 µmol/g (*3*). If liver reserves elicit a biological response to a vitamin A challenge dose, vitamin A status is not in equilibrium and the individual should be considered at risk for vitamin A deficiency.

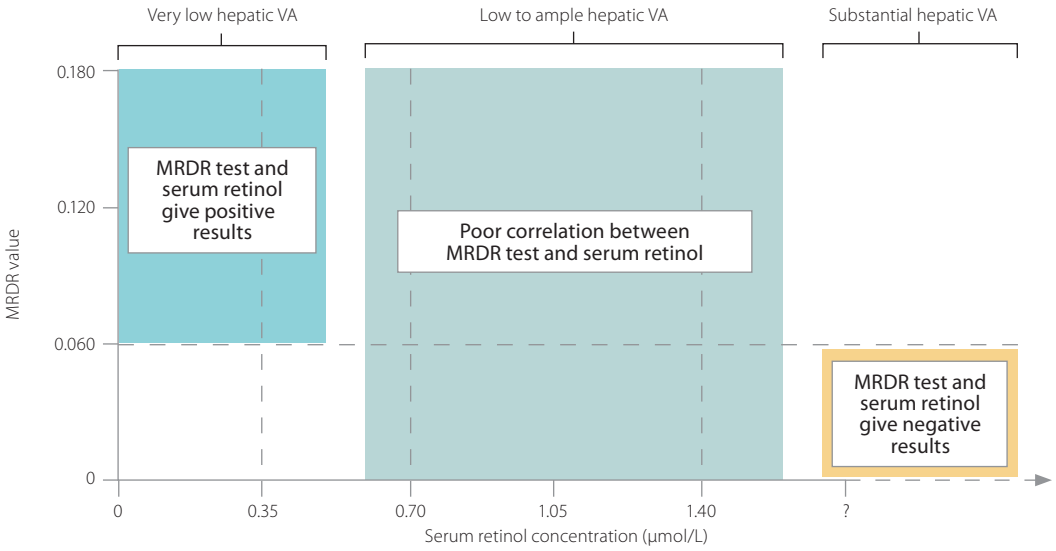
Figure A2.2.3
The relationship of the modified relative dose response (MRDR) value to liver retinol concentration in piglets. Below 17 µg/g liver the MRDR value is invariably positive, i.e. >0.060. Between 17 and 29 µg/g the response is split and above 29 µg/g liver the MRDR value is usually <0.060.



Data from references (*32*, *36–38*).

In practice, the MRDR and serum retinol concentrations will be in agreement when the serum retinol concentrations are either <0.5 µmol/L for defining vitamin A deficiency or >1.6 µmol/L for defining vitamin A adequacy (*38*). Many population groups fall into this grey area where the MRDR will be more descriptive than serum retinol concentrations alone (**Figure A2.2.4**).

Figure A2.2.4
Regions where serum retinol concentrations and modified relative dose response (MRDR) values will be highly correlated when used as indicators of vitamin A deficiency. VA, vitamin A. These are only applicable when individuals are free of infection and other confounders such as protein-energy undernutrition or zinc deficiency. A positive response means the indicator classifies the individual as vitamin A deficient while a negative response means the indicator classifies the individual as vitamin A adequate.



Retinol isotope dilution

The most sensitive method of evaluation of vitamin A status to liver reserves of vitamin A is isotope dilution testing (*40–42*), which uses either deuterated or ^{13}C-labelled retinyl acetate as the tracer. The deuterated retinol test uses conventional gas chromatography-mass spectrometry (GCMS), whereas the ^{13}C-retinol test uses gas chromatography-combustion-isotope ratio mass spectrometry (GCCIRMS). GCMS with electron capture negative chemical ionization detection has been found to increase the sensitivity of the method (*43*, *44*), but ^{13}C-retinol with GCCIRMS requires a smaller dose to trace total body stores (*41*, *45*).

All methods that calculate total body stores of vitamin A use the same fundamental mass-balance equation with various adaptations to reflect the unique metabolism of vitamin A (*46*):

$$(F_a \times a) + (F_b \times b) = (F_c \times c)$$

Where: a refers to the amount of dose absorbed and stored (determined experimentally to be 0.5–0.8 of that administered)

b is baseline reserves of vitamin A

c = a + b = total post dose reserves of vitamin A

$$F = \frac{R}{R+1} \quad \text{and R is } {}^{13}C/{}^{12}C$$

Paired-isotope dilution tests evaluate baseline and follow-up liver reserves to determine changes in response to an intervention (*47*, *48*). Examples of this include estimating relative vitamin A equivalency factors (*49*), assessing the effect of different intake levels of vitamin A on calculations of total body reserves (*50*) and effects of supplementation on liver stores (*44*). Vitamin A supplementation and abrupt changes in dietary intake may result in the exaggeration of liver reserves or affect value estimates with the test (*44*, *50*). The ^{13}C-retinol dilution test has been validated in rats with a depleted and adequate vitamin A status (*17*) and in rhesus monkeys known to have hypervitaminosis A against liver reserves (*51*). Measured versus predicted liver

reserves in these monkeys revealed a linear relationship and all the monkeys were diagnosed as having hypervitaminosis A by the predicted values (*41*).

Although isotope dilution testing is usually too expensive to consider as a means to evaluate a programme, a sugar-fortification programme was evaluated in a small group of Nicaraguan children (*52*). The baseline mean liver retinol concentration was 0.57 μmol/g liver, well above what is currently considered deficient (0.07 μmol/g liver). All the children had serum retinol concentrations between 0.74 and 1.31 μmol/L. One year after sugar fortification was implemented, liver reserve concentrations increased to an average of 1.2 μmol/g liver. In 9 of 21 children, liver vitamin A concentrations were calculated to be >1.05 μmol/g liver after fortification, which was defined as toxic in 1990 (*53*). Because many foods are now being considered for fortification, this sensitive methodology may have to be used, as no other method except liver biopsy is able to diagnose hypervitaminosis A.

Considering the validation in monkeys and these results in children, isotope methodology can be useful in defining the hypervitaminotic range of liver reserves. Specifically, liver reserves >10 μmol/g have been quantified (*41*). The ramifications of a sub-toxic or toxic vitamin A status in humans are largely not known. Excessive liver reserves have been defined as 0.70–1.05 μmol/g liver and toxic as >1.05 μmol/g in humans (*53*). However, after sugar fortification in Nicaragua, many of the children had liver reserves greater than this range (*52*). The liver vitamin A concentration at which ill health in humans occurs needs to be examined more carefully. Are there ramifications from having a liver reserve that is hovering around 1 μmol/g liver or is the human body able to sequester this level in the liver? Considering the degree of fortification in some developing countries, the improvements in the stability of the fortificants used in formulations and the high consumption of some of these fortified foods, there is a need for further examination of toxicity or hypervitaminosis A.

Discussion and conclusions

Biomarkers of vitamin A status are needed in order to more specifically identify populations at risk for vitamin A deficiency and to evaluate the effectiveness of different interventions. A variety of biomarkers exist because of the multiple functions of vitamin A in the human body. Some biomarkers are more sensitive to changes in liver vitamin A reserves than others. Serum retinol is affected by a number of factors including infection, inflammation and recent dietary intake. The dose response tests are less affected by infection. Serum retinol concentrations and the MRDR test are correlated when serum retinol concentrations are very low or very high. Combining biomarkers will be more descriptive than a single marker in a population. For example, evaluating a group of preschool children in a country may be better described if RBP measurements are taken from a stratified population-representative sample. Then a subset of children could undergo a more robust test, such as the MRDR or isotope dilution, to better describe the RBP distribution. Considering the degree of fortification of commonly consumed foods in some countries, more sensitive methodology, such as isotope dilution, may be needed in the future to evaluate the hypervitaminotic range of liver reserves in population groups.

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