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Reliability of Serum Assays of Iron Status in Postmenopausal Women

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Abstract

PURPOSE—The aim of the study is to determine the reliability during a 2-year period of several newly developed iron-related assays to assess their potential for use in prospective epidemiologic studies.

METHODS—We assessed the temporal reliability of several iron-related assays by using three serum samples collected at yearly intervals from 50 postmenopausal participants in a large prospective study.

RESULTS—We observed high reliability coefficients for ferritin (0.78; 95% confidence interval [CI], 0.67–0.86), soluble transferrin receptor (sTfR; 0.79; 95% CI, 0.69–0.87), sTfR/ferritin ratio (0.74; 95% CI, 0.62–0.83), and hepcidin (0.89; 95% CI, 0.84–0.94). In a subset of 30 women, lower reliability was observed for serum iron (0.50; 95% CI, 0.29–0.70), unsaturated iron-binding capacity (0.55; 95% CI, 0.34–0.73), total iron-binding capacity (0.60; 95% CI, 0.40–0.76), and serum transferrin saturation rate (0.44; 95% CI, 0.22–0.65). The reliability of anti-5-hydroxymethyl-2′-deoxyuridine autoantibody titers, a biomarker of oxidized DNA damage, one of the mechanisms by which iron is thought to impact disease risk, was very high (0.97, 95% CI, 0.5–0.99).

CONCLUSIONS—Our results show that some newly developed iron-related assays could be useful tools to assess iron–disease associations in prospective cohorts that collect a single blood sample.

Keywords

Ferritin; Hepcidin; Intraclass Correlation Coefficient; Iron; Transferrin Receptor; Reliability

INTRODUCTION

It was suggested that iron, which is involved in many metabolic processes, such as enzyme functions and DNA synthesis, has a role in the development of some chronic diseases, such as diabetes and cancer (1–3). The epidemiologic design of choice to study iron–disease associations is a prospective design because the presence of disease may affect iron levels in traditional case–control studies. Although some other biomarker characteristic, such as minimum/maximum or change over time, may be of interest, for diseases with a long

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development period, including cancer, the relevant exposure often is the biomarker's "usual" or average long-term level, rather than the level at a single point in time (4,5). Large prospective studies often collect biologic samples on all (or most) participants at only one point in time (6–8). Using a single measurement to characterize a subject's usual level introduces measurement error that will lead to an observed exposure–disease association that will differ from the true underlying association. In many, but not all, cases, the association will be attenuated (9). Thus, in addition to reflecting components of iron status relevant to a specific disease process, a biomarker needs to be reliable, i.e., a subject's measurement at a single point in time needs to reflect reasonably well this subject's long-term average level relative to other subjects for the assay to provide valid information on iron–disease associations (9,10). It therefore is important before undertaking a large epidemiologic study of biomarkers to assess the reliability of these biomarkers.

Some relatively new assays are available to measure various biomarkers of iron stores, such as transferrin receptor (TfR; a membrane protein involved in iron incorporation into cells) and hepcidin (a protein that downregulates iron stores by inhibiting both duodenal absorption and macrophage release of iron) (11,12). We conducted a study of postmenopausal women to assess the reliability of these and other iron assays during a 2-year period by using samples from the New York University (NYU) Women's Health Study, a prospective cohort in which a subset of participants donated repeated samples (13,14). We assayed three serum samples collected at yearly intervals from 50 postmenopausal women for ferritin, soluble TfR (sTfR), and hepcidin. We also assessed serum iron (SI), serum unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and transferrin saturation in a subset of 30 women. Finally, we assessed the reliability of anti-5-hydroxymet hyl-2′-deoxyuridine (HMdU) autoantibody (aAb) titers, a biomarker suggested to reflect oxidized DNA damage (15), because induction of oxidative damage is one of the main mechanisms by which iron is thought to have a role in the development of chronic diseases (16).

METHODS

NYU Women's Health Study

Between March 1985 and June 1991, the NYU Women's Health Study enrolled a cohort of 14,275 healthy women aged 34 to 65 years at a breast cancer screening center in New York City (13,14). Women who had been pregnant or administered hormone medications in the 6 months preceding their visit were not eligible. At the time of enrollment and at annual screening visits thereafter, women were asked to donate blood and complete a self-administered questionnaire. Thirty milliliters of nonfasting peripheral venous blood was drawn before breast examination. After blood drawing, tubes were kept covered at room temperature (21°C to 25° C) for 15 minutes and at 4°C for 60 minutes to allow clot retraction, then centrifuged for 25 minutes. After centrifugation, serum samples were divided into 1-mL aliquots and immediately stored at −80°C for future biochemical analyses. Approximately half the participants gave blood at more than one visit (mean number of blood donations, three; range, two to eight). Women have since been followed-up for cancer and cardiovascular end points. Nested case–control studies of breast, colon, ovarian, and endometrial cancer, as well as coronary heart disease, are ongoing.

Sample Selection

Initially, 30 women were selected at random from the pool of eligible women. This pool consisted of the NYU Women's Health Study participants who were postmenopausal at entry, had given blood on three or more occasions at yearly intervals, had a yield of 11 or more aliquots at each visit, had not had a diagnosis of cancer or cardiovascular disease, and had not been selected as a control in any case–control study nested within the cohort. For each woman, SI,

UIBC, TIBC, transferrin saturation rate, and HMdU aAb were measured in three yearly samples. Before assessing the more recent iron biomarkers (ferritin, sTfR, and hepcidin), an additional 20 women were selected from the pool of eligible women (for a total of 50 women) to increase the precision of reliability coefficient estimates.

Laboratory Analyses

Serum samples were identified solely by a sample number so that laboratory personnel were not aware of the identity of contributing participants. All samples from a subject always were assayed in the same batch. For 20 women included in the study, a second aliquot from one of the three blood donations also was assayed after relabeling with a new sample number to ensure blinding. These blinded quality-control samples were used for assessment of the intrabatch coefficient of variation.

Preparation of serum samples, as well as techniques for determining ferritin, SI, UIBC, TIBC, transferrin saturation rate (percent), and HMdU aAb titers, were described previously (15,17, 18). sTfR in sera was determined by using an enzyme-linked immunosorbent assay technique using two different monoclonal antibodies specific for sTfR (R & D System, Minneapolis, MN). After determining sTfR and ferritin levels, the molar ratio of sTfR to ferritin was calculated. Levels of hepcidin were determined by using a newly developed enzyme-linked immunosorbent assay based on a competitive principle (DRG International Inc., Mountainside, NJ). Fifty microliters of serum samples were used for this assay.

Statistical Methods

Reliability was estimated by using the intraclass correlation coefficient assuming a one-way random-effects analysis of variance model. Estimation was carried out by using restricted maximum-likelihood techniques in SAS PROC VARCOMP (SAS Institute, Cary, NC). Computations were performed on $\log_e$-transformed data.

RESULTS

Women had a median age at first blood donation of 62.3 years (range, 49.5 to 68.1 years), with a median time since menopause of 12.6 years (range, 2.2 to 25.3 years). Median weight and body mass index were 66.0 kg (range, 43.0 to 86.0 kg) and 24.6 kg/m^2 (range, 19.2 to 35.9 kg/m^2), respectively. Serum samples were in storage at −80°C for a median of 15.6 years (range, 14.8 to 17.1 years).

Table 1 lists intrabatch coefficients of variation, medians and ranges, and reliability coefficients for all measurements. Hepcidin had the highest coefficient of variation (15%), probably because of the competitive nature of the assay, an indirect way to measure hepcidin. For all other assays, coefficients of variation were 11% or less. Precision of the assays did not appear to vary greatly according to levels of biomarkers: coefficients of variation for greater than versus less than the median value of all observed measurements (Table 1) were 11% and 8% for ferritin, 4% and 5% for sTfR, 9% and 13% for sTfR/ferritin molar ratio, and 16% and 14% for hepcidin, respectively. For all other assays, coefficients of variation were similar for greater than versus less than median levels of all measurements. Levels were within the expected range for women in this age group (1,11,15,19). Reliability coefficients were high for ferritin (0.78; 95% confidence interval [CI], 0.67–0.86), sTfR (0.79; 95% CI, 0.69–0.87), sTfR/ferritin ratio (0.74; 95% CI, 0.62–0.83), and hepcidin (0.89; 95% CI, 0.84–0.94). The other iron assays had lower reliability coefficients, varying from 0.44 to 0.60. Anti-HMdU antibody assay had very high reliability of 0.97 (95% CI, 0.95–0.99).

DISCUSSION

Iron may contribute to the development of diseases through its oxidative properties (20). Iron tightly bound to proteins is not readily bioavailable for oxidative reactions, but iron bound to such low-molecular-weight (LMW) chelators as citrate and adenosine triphosphate can generate oxidants through Fenton/Haber–Weiss or autoxidation reactions that may lead to damage of cellular membranes, proteins, and DNA (21). Measurement of circulating LMW iron therefore would be of great interest. Unfortunately, there currently is no method sensitive enough to measure the low levels of LMW iron present in serum of apparently healthy subjects (22,23). However, a number of assays are available that are indicative of various aspects of iron status. We assessed the reliability of some of these assays because the ability of a single subject's measurement to reflect this subject's long-term level reasonably well is a prerequisite for epidemiologic studies with access to a single biologic sample per subject to provide valid information on iron–disease associations.

In our study, we found that serum levels of ferritin, a storage protein with a capacity of binding up to 4500 atoms of iron per molecule of ferritin, remain stable over time in postmenopausal women. This result is in agreement with previous studies (17,24–26). The high reliability of circulating ferritin coupled with its positive correlation with residual iron stores, measured by using quantitative phlebotomy (27), makes it a valuable assay in epidemiologic studies.

TfR is a membrane protein that forms a complex with transferrin, an iron transport protein, before incorporation of iron into cells (28). During the process of recycling, some TfRs are shed and appear as sTfRs that can be measured by using a recently developed assay (29). Like ferritin, sTfR was found to be a reliable biomarker that could be used in epidemiologic studies.

sTfR/ferritin ratio also was used in epidemiologic studies (1). It had a close inverse loglinear relationship with body iron stores expressed per kilogram of body weight, and it may be more sensitive to deficits in functional iron than ferritin alone (12,30). Synthesis of ferritin and TfR is regulated by LMW iron through iron-responsive element-binding protein (IRE-BP). In the setting of low levels of LMW iron, IRE-BP binds to a 3′-regulated untranslated region in TfR messenger RNA (mRNA), increasing mRNA stability and leading to increased transcription and a greater number of TfRs. Conversely, IRE-BP binds to the 5′ untranslated region of ferritin mRNA and thus inhibits the translation of ferritin mRNA, leading to a decreased ferritin level (31). The sTfR/ferritin molar ratio therefore may have greater ability to characterize a subject's serum level of labile and potentially toxic iron than ferritin alone, with a high sTfR/ferritin ratio reflecting a low level of LMW iron and a low ratio reflecting an iron overload.

The reliability coefficient for hepcidin was high in our study, making this recently discovered protein that regulates iron stores through inhibition of both iron absorption in the duodenum and iron release from macrophages (32) another potential tool for epidemiologic studies.

Circadian and day-to-day variations were reported previously for serum iron levels (11). It therefore is not surprising that the reliability of serum iron level based on samples collected at yearly intervals and varying times of the day is fairly low (0.50; 95% CI, 0.29–0.70). Because serum iron enters in calculations of TIBC and transferrin saturation rate, the long-term reliability of these measurements also would be expected to be limited, as observed.

It is possible that recent food intake affects levels of some iron biomarkers (33). Subjects in our study were not required to fast before blood donation. This suggests that for the biomarkers for which we observed high reliability coefficients, the effect of fasting is at most moderate.

Controlling for factors that affect iron stores likely would further increase biomarker reliability. Some of these factors did not apply to our study, e.g., menses, pregnancy (34) (because our

study focused on postmenopausal women), and use of hormone replacement therapy (35) (because this was an ineligibility criterion). Another factor that affects iron marker levels is occult bleeding, e.g., from a gastrointestinal ulcer (35); however, the prevalence of occult bleeding is expected to be low in a population of healthy women, such as our cohort (33). In postmenopausal women, ferritin levels seem to increase with age until approximately 60 years and then reach a plateau (35–37). Iron supplement intake increases ferritin levels (35,38), although in the largest study (33), this effect was limited to intake greater than 32 mg/d of iron (more than four times the recommended dietary allowance). Other factors reported to affect iron marker levels in postmenopausal women include blood donation, aspirin use, intake of heme iron, body mass index, physical activity, and alcohol intake (35).

Iron-catalyzed oxidants may cause DNA damage. Typical immediate results of oxidative attack on DNA include formation of 5-HMdU, an oxidized DNA base derivative. It appears that the presence of 5-HMdU in DNA stimulates production of a specific immunoglobulin M–class autoantibody, suggesting that these antibodies may constitute a marker of oxidative DNA damage (39). The very high reliability of the HMdU aAb assay makes it a good candidate assay for epidemiologic studies.

Although a role for iron in a number of chronic diseases has long been suggested, the number of epidemiologic studies conducted is limited and results have not been consistent. This may be caused, at least in part, by the modest temporal reliability of older assays (SI, UIBC, TIBC, and serum transferrin saturation rate), as we observed in our study. The availability of new assays measuring various aspects of iron metabolism offers the opportunity to reexamine the role of iron in chronic diseases. Our results indicate that serum ferritin and sTfR levels, their molar ratio, and hepcidin levels were stable during a 2-year period in postmenopausal women. Hence, these biomarkers can be useful tools to assess anew associations of chronic diseases with iron in prospective epidemiologic studies that collected a single blood sample.

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Selected Abbreviations and Acronyms

aAb	autoantibody
HMdU	5-hydroxymethyl-2′-deoxyuridine
IRE-BP	iron-responsive element-binding protein
LMW	low molecular weight
NYU	New York University
SI	serum iron
sTfR	soluble transferrin receptor
TfR	transferrin receptor
TIBC	total iron-binding capacity
UIBC	unsaturated iron-binding capacity
CI	confidence interval

mRNA messenger RNA

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TABLE 1

Intrabatch coefficients of variation, median, range, and reliability coefficients of assays

Assays	Intrabatch coefficient of variation, %	Median (range)	Reliability coefficient[a] (95% confidence interval)
Assays conducted in 50 women			
Ferritin, nmol/L	9	2.76 (0.32–7.16)	0.78 (0.67–0.86)
Soluble transferrin receptor, nmol/L	4	22.4 (7.8–68.5)	0.79 (0.69–0.87)
Soluble transferrin receptor–ferritin molar ratio	11	7.8 (0.28–134.2)	0.74 (0.62–0.83)
Hepcidin, ng/mL	15	243.4 (37.1–1667)	0.89 (0.84–0.94)
Assays conducted in 30 women			
Serum iron, μmol/L	10	15.7 (5.0–36.3)	0.50 (0.29–0.70)
Unsaturated iron-binding capacity, μmol/L	2	37.5 (20.9–71.6)	0.55 (0.34–0.73)
Total iron-binding capacity, μmol/L	5	52.1 (38.6–100.1)	0.60 (0.40–0.76)
Serum transferrin saturation rate, %	6	28.6 (8.0–54.0)	0.44 (0.22–0.65)
Anti-5-hydroxymethyl-2′-deoxyuridine autoantibody titer, A_{492}/μL	3	11.7 (1.4–41.6)	0.97 (0.95–0.99)

[a] On $\log_e$-transformed data.