Assessing the IRON STATUS of populations

Second edition

Including Literature Reviews

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Contents

Report of a Joint World Health Organization/
Centers for Disease Control and Prevention

Technical Consultation on the Assessment of Iron Status at the Population Level

GENEVA, SWITZERLAND
6–8 APRIL 2004

1. Consultation

1.1 Rationale for the Consultation

Anaemia is one of the most common and intractable nutritional problems in the world today. The World Health Organization (WHO) estimates that some two billion people are anaemic defined as haemoglobin concentrations that are below recommended thresholds. The main causes of anaemia are: dietary iron deficiency; infectious diseases such as malaria, hookworm infections and schistosomiasis; deficiencies of other key micronutrients including folate, vitamin B_{12} and vitamin A; or inherited conditions that affect red blood cells (RBCs), such as thalassaemia.

Iron deficiency with or without anaemia has important consequences for human health and child development: anaemic women and their infants are at greater risk of dying during the perinatal period; children's mental and physical development is delayed or impaired by iron deficiency; and the physical work capacity and productivity of manual workers may be reduced. There have been many efforts to fight iron deficiency and anaemia over the past two decades but, despite these efforts, the conditions are still common.

One of the reasons for the apparent failure to reduce the prevalence of anaemia is that many programmes and their interventions have been designed with the assumption that the only cause of anaemia is iron deficiency. This has meant that, when trying to control anaemia, the role of other causes has been underestimated, and that iron deficiency without anaemia has not been addressed as a major and common health problem.

In the absence of international agreement on how to assess the iron status of populations, the prevalence of iron deficiency has often been derived from the prevalence of anaemia using measurements of blood haemoglobin concentration. However not all anaemic people are iron deficient and iron deficiency may occur without anaemia. This means that the prevalence of anaemia and iron deficiency varies in different populations and no consistent relationship between the two can be applied throughout the world. When anaemia is considered from the point of view of programmes to improve nutrition and health, an estimate of the prevalence derived from the haemoglobin concentration alone does not allow the contribution of iron deficiency to anaemia to be estimated, and ignores the role of other causes of anaemia.

To plan effective interventions to combat both iron deficiency and anaemia there is an urgent need to have better information on the iron status of populations. This will enable the right interventions to be chosen in the first place and then, once programmes are in place, to have the right indicators to monitor their impact.

These were all reasons for holding the Joint WHO/Centers for Disease Control and Prevention (CDC) Technical Consultation on the Assessment of Iron Status at the Population Level. The Consultation took place in Geneva, Switzerland, from 6 to 8 April 2004.

1.2 Objectives of the Consultation

The objectives of the Consultation were:

- to review the indicators currently available to assess iron status;
- to select the best indicators to assess the iron status of populations;
- to select the best indicators to evaluate the impact of interventions to control iron deficiency in populations;

- to identify priorities for research related to assessing the iron status of populations.

2. Working definitions of key terms

For the sake of clarity and to achieve a consensus, several key terms were defined during the Consultation.

In clinical terms *anaemia* is an insufficient mass of RBCs circulating in the blood; in public health terms *anaemia* is defined as a haemoglobin concentration below the thresholds given by WHO, UNICEF, UNU (*1*). These thresholds are set at the 5th percentile of the haemoglobin concentration of a normal population of the same sex and age group. There is a separate threshold for pregnant women.

Although iron deficiency is probably the most common cause of anaemia, there are other causes as well, including: acute and chronic infections that cause inflammation; other micronutrient deficiencies, especially of folate, vitamin B_{12} and vitamin A; and genetically inherited traits such as thalassaemia.

Iron deficiency is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as the blood, brain, and muscles. Iron deficiency can exist in the absence of anaemia if it has not lasted long enough or if it has not been severe enough to cause the haemoglobin concentration to fall below the threshold for the specific sex and age group (*1*). Evidence from animals fed on iron-deficient diets indicates that iron deficiency becomes detectable at about the same time in the blood, brain, and tissue enzyme systems (*2*).

Storage iron is the pool of iron in the body that is not being used by tissues. Healthy children and adults (apart from infants aged 6–11 months and pregnant women) usually have some iron stores to act as a buffer against iron deficiency during periods when dietary iron may be temporarily insufficient. *Iron depletion* is the state in which storage iron is absent or nearly absent but the tissues that need iron are able to maintain normal physiological functions.

It is possible for a *functional iron deficiency* to develop even when iron stores are present if the normal physiological systems for transporting iron to target tissues are impaired. This occurs most commonly because of cytokines released during inflammation caused by infectious diseases, and appears to be mediated by hepcidin (*3*). Iron supplementation or fortification has no benefit in such circumstances. Deficiencies of other nutrients such as vitamin A may also cause a functional iron deficiency even when iron stores are adequate (*4*).

3. Selection of indicators

In January 2004 a WHO/CDC working group met to review the literature on indicators of iron status and to select the indicators considered to be the best for discussion by the Consultation. Appendix 1 shows the indicators considered, each of which was evaluated in terms of its theoretical advantage as an indicator of iron status and the practicality of its measurement. Appendix 2 shows the five indicators selected to assess iron status and an acute phase protein with the methods most commonly used to measure them. The rationale for selecting these five was as follows.

- Haemoglobin concentration is a measure of anaemia, a condition that has important outcomes for health and child development that are linked to international development goals.

- Zinc protoporphyrin reflects a shortage in the supply of iron in the last stages of making haemoglobin so that zinc is inserted into the protoporphyrin molecule in the place of iron. Zinc protoporphyrin can be detected in RBCs by fluorimetry and is a measure of the severity of iron deficiency.
- Mean cell volume indicates whether RBCs are smaller than usual (microcytic), which is a common sign of iron deficiency anaemia, or larger than normal (macrocytic), a common sign of megaloblastic anaemia resulting from a deficiency of vitamin B_{12} or folate.
- Transferrin receptor in serum is derived mostly from developing RBCs and so reflects the intensity of erythropoiesis and the demand for iron; the concentration rises in iron deficiency anaemia and it is a marker of the severity of iron insufficiency only when iron stores have been exhausted, provided that there are no other causes of abnormal erythropoiesis. The concentration of transferrin receptor is also increased in haemolytic anaemia and thalassaemia. Clinical studies indicate that the serum transferrin receptor is less affected by inflammation than serum ferritin (*5*).
- Serum ferritin is a measure of the amount of iron in body stores if there is no concurrent infection: when the concentration is ≥15 µg/l iron stores are present; higher concentrations reflect the size of the iron store; when the concentration is low (<12–15 µg/l) then iron stores are depleted. When infection is present the concentration of ferritin may increase even if iron stores are low; this means that it can be difficult to interpret the concentration of ferritin in situations in which infectious diseases are common.

4. Literature reviews

The Consultation was provided with literature reviews on indicators of iron status, including RBC parameters, ferritin, free erthyrocyte protoporphyrin, serum and plasma iron, total iron binding capacity, transferrin saturation and serum transferrin receptor as well as a review on the interpretation of indicators of iron status during an acute phase response. These reviews provide technical background to the measurement, biology, interpretation and diagnostic value of the indicators. See annexes.

5. Analysis of data from iron intervention studies

In order to assess the potential of indicators to detect a change in iron status as a result of an intervention, the Consultation reviewed the results of an analysis of indicators of iron status and acute phase proteins that were measured during 10 double-blind, randomized controlled trials. The investigators provided iron either as supplements or as food fortified with iron for periods between 4 and 18 months to infants (1 study), preschool children (1 study), schoolchildren (2 studies), pregnant women (2 studies) and non-pregnant women (4 studies). The studies were done in Côte d'Ivoire (*6*), Jamaica (Simmons et al., unpublished data), Morocco (*7*), the Philippines (Beard and Haas, unpublished data), one study done in Sweden and Honduras (*8*), one study in the United Republic of Tanzania (*9*), two studies in the United States of America (Beard, unpublished data and *10*), and two studies in Viet Nam (Thuy et al., unpublished data and *11*). The original data sets from all trials were provided for this analysis by the investigators, who are acknowledged at the end of this report.

Full details of the analysis will be submitted for publication in due course.

The haemoglobin and serum ferritin concentrations were measured in all ten trials, serum transferrin receptor in nine, zinc protoporphyrin in six, and mean cell volume by flow cytometry in four. For the nine studies that measured both serum ferritin and transferrin receptor, the body iron stores were estimated using the method and constants given by Cook, Flowers, Skikne (*12*). Because both serum ferritin and transferrin receptor concentrations have distributions skewed to the right, both raw values and values transformed to logarithms were used in the analysis.

5.1 Indicators for evaluating the impact of interventions to control iron deficiency

The data from the ten studies were selected because the experimental designs were considered to be adequate (duration of intervention, iron dosage and compound) to show an improvement in iron status. The data were used to assess how well each indicator predicted the changes in iron status. This change was estimated by calculating the mean difference between each indicator at the beginning and end of each study for the intervention group and then subtracting the mean difference calculated for the control group. As each indicator had different units, the net difference between the change in the intervention and control groups was divided by the standard deviation of the baseline measurement of the indicator calculated for both study groups in order to express it in standard deviation units (SDUs). For the purposes of analysis an arbitrary change of ≥0.2 SDUs was defined as indicating a successful response to the intervention. A power calculation indicated that this change could be detected with a sample size of 400 subjects per study group.

In addition, using the same studies, the change that occurred in the subjects with the lowest 10% of values was examined for indicators that were expected to rise, such as haemoglobin concentration, and for the highest 10% of values for indicators that were expected to fall, such as transferrin receptor. The change in values of these 10th or 90th percentiles was also expressed as SDUs. This analysis was based on the assumption that the most iron deficient subjects would show the greatest change as a result of the interventions. Table 1 shows how the indicators performed when assessed in these two ways.

An analysis of the effect of using different thresholds of success varying between 0.1 and 0.5 SDUs did not change the inferences about each indicator in each study.

TABLE 1

Success of indicators to detect changes in iron status in 10 controlled trials of treatments, estimated as the number of indicators showing a change of ≥0.2 standard deviation units (SDUs)

Indicator of iron status	Success of indicator based on mean change of ≥0.2 SDUs for all subjects	Success of indicator based on mean change of ≥0.2 SDUs for top or bottom 10%[a]
Haemoglobin	60%, 6 of 10 studies	80%, 8 of 10 studies
Mean cell volume	50%, 2 of 4 studies	75%, 3 of 4 studies
Serum ferritin[b]	90%, 9 of 10 studies	60%, 6 of 10 studies
Transferrin receptor[c]	56%, 5 of 9 studies	56%, 5 of 9 studies
Body iron stores	78%, 7 of 9 studies	78%, 7 of 9 studies
Zinc protoporphyrin	50%, 3 of 6 studies	67%, 4 of 6 studies

[a] Depends on whether the indicator was expected to rise or fall.
[b] Transformed to logarithms.
[c] Results were the same with or without transforming values to logarithms.

In the one study in which serum ferritin did not meet the criterion for success based on the change for all subjects, neither did any other indicator. The indicator of body iron stores calculated from serum ferritin and transferrin receptor did not perform better than serum ferritin alone. It was not possible to distinguish between the performance of haemoglobin, transferrin receptor, zinc protoporphyrin and mean cell volume.

The analysis was based on trials that had a control group to account for secular and random change. However, many programmes simply evaluate differences between before and after an intervention, and do not have a control group in the design. To examine whether different indicators would be selected in the absence of a control group, two questions were asked.

First, in how many studies did the value of the indicator change in the control group by ±0.2 SDUs? A change of this size in either direction would imply that the indicator is not very stable. It could also indicate an additional source of iron other than the intervention, or a change in exposure to hookworms or malaria, that resulted in a change in iron status. Second, in how many studies would the wrong inference have been made if there had been no control group to account for secular or random changes?

Four studies were excluded from these analyses because they involved pregnant women or young children among whom changes would have been expected anyway, without any intervention. Of the remaining six studies, which were done among schoolchildren or non-pregnant women, mean cell volume was only measured in one study and zinc protoporphyrin in two. Table 2 shows the results of this analysis, which indicate that serum ferritin and body iron stores performed best.

TABLE 2
The results of an analysis of the stability of indicators of iron status in control groups during studies of interventions and of the inferences that would have been wrong without a control group

Indicator of iron status	Number of studies in which there was a significant change in the control group of ± 0.2 SDUs	Number of studies in which the inference would have been wrong without a control group
Haemoglobin	4 of 6	2 of 6
Serum ferritin	1 of 6	1 of 6
Transferrin receptor	3 of 6	2 of 6
Body iron stores	1 of 6	1 of 6
Zinc protoporphyrin	2 of 2	1 of 2

SDUs, Standard deviation units.

Based on results of the analysis presented in Table 1 and 2, it was concluded that serum ferritin is the indicator of choice to evaluate the impact of interventions to control iron deficiency in studies with or without control groups.

5.2 Performance of indicators to predict a change in haemoglobin concentration in response to iron intervention

A critical issue for the Consultation was to decide which current indicator represents the best means to identify a true iron deficiency and could act as the "gold standard" by which to evaluate alternative indicators. The change in haemoglobin concentration following intervention using iron was chosen based on the assumption that the size of any change was likely to be strongly related to the degree of iron deficiency.

This measure has been used in previous studies (*13*). It has a disadvantage because if factors other than iron deficiency contribute to anaemia, such as a vitamin A deficiency, then the haemoglobin concentration will not respond to treatment with iron alone.

A linear regression analysis was performed to examine the degree to which baseline indicators of iron status predict a change in haemoglobin, using the following model:

$$Y = \beta_0 + \beta_1 * X_1 + \beta_2 * X_2 + \beta_3 * X_1 X_2 + \varepsilon$$

Where: Y is the change in haemoglobin concentration from baseline to follow-up

X_1 is the group (control versus intervention)
X_2 is the baseline iron indicator measured in SDUs
X_1X_2 is the interaction between group and iron indicator
ε is the error or residual.

The coefficient of interest in this analysis is (β_3) defined as the excess change in haemoglobin concentration for intervention over control for each additional SDU of the selected iron indicator at baseline. A statistically significant interaction term (β_3) was interpreted to mean that the indicator was associated with a change in haemoglobin concentration in response to intervention with iron, whether given as supplements or as fortified food. An indicator was arbitrarily classified as successful if there was an increase in haemoglobin concentration by ≥3 g/l for each SDU of the selected indicator at baseline. An advantage of this approach is that it uses the control group to take into account both secular trends in haemoglobin concentration and regression to the mean. Variables that were not normally distributed were transformed to logarithms to see if their predictive power could be improved.

Table 3 shows the success of each indicator in predicting the change in haemoglobin concentration in response to an iron intervention. Using an increase of 2–5 g/l made no difference to the results of the analysis.

Table 3 shows that the indicators were generally less successful in predicting the response of the haemoglobin concentration to treatment with iron than in assessing the response to interventions shown in Table 1. In fact in six of the ten studies, including the three studies in Africa, none of the indicators were associated with the change in haemoglobin concentration. This could have been because low haemoglobin concentrations were caused by factors other than iron deficiency, such as infectious diseases, which impaired the haemoglobin response to the interventions. Nevertheless, this analysis led to the following tentative conclusion, that serum ferri-

TABLE 3
The success rate of indicators of iron status to predict an increase in haemoglobin of ≥3 g/l for each standard deviation unit of the indicator at baseline

Indicator of iron status	Success rate
Haemoglobin	10%, 1 of 10 studies
Mean cell volume	25%, 1 of 4 studies
Serum ferritin[a]	40%, 4 of 10 studies
Transferrin receptor	33%, 3 of 9 studies
Body iron stores	44%, 4 of 9 studies
Zinc proto porphyrin	17%, 1 of 6 studies

[a] Transformed to logarithms.

tin or transferrin receptor are the best indicators to predict a change in haemoglobin concentration in response to iron intervention and, if both indicators are measured, then body iron stores can be estimated as well. But these indicators were successful in less than half of the studies analysed.

6. Indicators of inflammation

The Consultation considered that serum ferritin was the best indicator of the impact of an iron intervention as well as being a useful indicator of depleted iron stores. However serum ferritin is also an acute phase protein, which means that its concentration rises during inflammation, so the customary thresholds to indicate an iron deficiency of <12–15 µg/l may no longer apply. One way of dealing with this issue is to set the threshold higher, and a threshold of <30 µg/l has been recommended in the presence of infection, but only for children <5 years old (*1*). There is a need to examine the value of using different thresholds among infected older children and adults.

The Consultation proposed that the measurement of an acute phase protein could help to interpret data on serum ferritin: if the concentration of the additional acute phase protein is higher than the normal threshold it could indicate underlying inflammation and explain a high serum ferritin concentration in the presence of iron deficiency.

One way of controlling for a high serum ferritin concentration resulting from infection would be to use the concentration of another acute phase protein to exclude individuals whose measurements of both indicators are above a certain threshold. This approach is not considered feasible in many parts of sub-Saharan Africa where many people are infected with *Plasmodium* spp., the cause of malaria, and are either asymptomatic or have only mild disease and yet have high concentrations of acute phase proteins in their blood (*14*). Many of the same individuals may also be chronically infected with one or more species of worms, which may also contribute to an acute phase response as well as to anaemia and iron deficiency because of the blood loss they cause. There may even be an acute phase response without a loss of blood. Excluding individuals with high concentrations of acute phase protein may, in circumstances in which repeated or chronic infections are common, reduce the sample size substantially and leave an atypical residual sample.

The Consultation felt that there was a need for the analysis of data on the relationship between serum ferritin, transferrin receptor and different acute phase proteins to assess which was best correlated with serum ferritin during different stages of infection. For example, it may be possible to control for high serum ferritin concentrations using one or more acute phase proteins. Several acute phase proteins could be used for this purpose including C-reactive protein (CRP), α-1-antichymotrypsin (ACT), α-1 acid glycoprotein (AGP), serum amyloid A, fibrinogen and haptoglobin. The most frequently used acute phase proteins are CRP, which responds quickly to inflammation but also subsides quickly in concentration; ACT which also rises quickly but remains at a high concentration longer than CRP; and AGP which is slower to respond than CRP or ACT but remains at a high concentration for longer than either (*15,16*). The concentration of AGP maybe a better indicator than CRP or ACT of the presence of chronic, sub-clinical infection, and may better reflect the changes in the concentration of ferritin during infections.

The Consultation proposed that data should be sought from studies in diverse settings that have, if possible, measured haemoglobin concentration, serum ferritin and transferrin receptor, and at least one acute phase protein, with CRP, ACT and AGP as the first choices. Because an assay for transferrin receptor has only relatively recently become available, data sets that do not include this measurement but have measured another acute phase protein as well as serum ferritin would also be useful. Data on the presence of infectious diseases or on malaria parasitaemia, and on the intensity of worm infections would also be helpful to examine the relationship between specific infections and acute phase proteins.

7. Recommendations

The Consultation made the following recommendations based on the analysis of studies presented, the literature reviews, and on the debates during the Consultation.

7.1 Assessing the iron status of populations

The concentration of haemoglobin should be measured, even though not all anaemia is caused by iron deficiency. The prevalence of anaemia is an important health indicator and when it is used with other measurements of iron status the haemoglobin concentration can provide information about the severity of iron deficiency.

Measurements of serum ferritin and transferrin receptor provide the best approach to measuring the iron status of populations. In places where infectious diseases are common, serum ferritin is not a useful indicator because inflammation leads to a rise in the concentration of serum ferritin as a result of the acute phase response to disease. If infectious diseases are seasonal, then the survey should be done in the season of lowest transmission. In general the concentration of transferrin receptor does not rise in response to inflammation so that, when combined with the concentration of serum ferritin, it is possible to distinguish between iron deficiency and inflammation. Table 4 indicates how data on serum ferritin and transferrin receptor may be interpreted based on the experience of participants of the Consultation. For the purposes of describing the prevalence of iron deficiency in a population with a single number, the prevalence based on serum ferritin should be used except where

TABLE 4
The interpretation of low serum ferritin and high transferrin receptor concentrations in population surveys: this classification is based on experience of measuring ferritin and transferrin receptor in research studies and requires validation in population surveys

Percentage of serum ferritin values below threshold[a]	Percentage of transferrin receptor values above threshold[b]	Interpretation
<20%[c]	<10%	Iron deficiency is not prevalent.
<20%[c]	≥10%	Iron deficiency is prevalent; inflammation is prevalent.
≥20%[d]	≥10%	Iron deficiency is prevalent.
≥20%[d]	<10%	Iron depletion is prevalent.

[a] Apply thresholds by age group given in WHO, UNICEF, UNU (*1*)
[b] Apply thresholds recommended by manufacturer of assay until an international reference standard is available (See Section 8, Priorities for research, below).
[c] <30% for pregnant women.
[d] ≥30% for pregnant women.

inflammation is prevalent (Table 4, row 2) in which case the prevalence based on transferrin receptor is more appropriate. However, the proposed classification still requires validation in population surveys.

Studies are needed to determine the best procedures to process, transport and store biological samples in which transferrin receptor will be measured, and to establish internationally applicable thresholds to classify the iron status of populations.

It can be useful also to measure the concentration of an acute phase protein, if funding is available. The most commonly measured acute phase protein is CRP, but there is evidence that AGP may better reflect the change in concentration of ferritin in serum and may be the most useful acute phase protein to measure. A number of commercial assays are available for measuring these proteins but, except for CRP, there are no international reference standards available, resulting in reference ranges specific to each assay. In such circumstances, the threshold recommended by the manufacturer should be used.

7.2 Evaluating the impact of interventions to control iron deficiency in populations

Serum ferritin is the best indicator of a response to an intervention to control iron deficiency and should be measured with the haemoglobin concentration in all programme evaluations. In circumstances in which iron deficiency is the major cause of anaemia, the haemoglobin concentration may improve more rapidly than the serum ferritin concentration. In circumstances in which the serum ferritin concentration improves (even when inflammation is common) but the haemoglobin concentration does not, factors in addition to iron are likely to be the cause of anaemia.

If funding is available, it could also be useful to measure the concentration of one or both of the acute phase proteins CRP or AGP, to account for a high serum ferritin concentration caused by inflammation. Individuals with high values for the acute phase protein should be excluded from the analysis, if possible, depending on the limitations imposed by the sample size of the dataset and the consequent translation of the results to define the iron status of the general population. This is particularly important when repeated surveys are done and there is no control group for the intervention.

If funding is available, the transferrin receptor should be measured during repeated surveys to classify populations according to the criteria shown in Table 4. The combination of serum ferritin and transferrin receptor may also be used to estimate body iron stores in populations (*12*). The calculation of body iron stores is not essential but can be useful to estimate the amount of iron that is absorbed during an intervention and to demonstrate a decrease in iron deficiency. However, since the method uses measurements of serum ferritin concentration, infection may again be a confounding factor, so an acute phase protein should be measured to exclude individuals with a high concentration.

A working group will be established to coordinate the analysis of data sets containing estimates of serum ferritin, transferrin receptor and acute phase proteins and to make suggestions about how to improve the assessment of iron status.

In three years time, another consultation will be held to evaluate the recommendations made here based on the results of recent studies of assessing iron status.

8. Priorities for research

- There is an urgent need for an international reference material with a certified concentration of transferrin receptor to standardize transferrin receptor assays.
- A review of existing data is needed to confirm the thresholds used in Table 4 to derive a classification of iron status. The analysis should also examine alternative approaches using serum ferritin alone, and with one or two acute phase proteins. Thresholds for other indicators of iron status, such as zinc protoporphyrin, should also be examined if possible.
- A review of existing data is needed to examine which acute phase proteins might best be used to interpret data on serum ferritin during both acute and chronic infections, and whether data on serum ferritin could be corrected rather than excluded. This review would identify the best acute phase protein to use and the thresholds to apply for both the acute phase protein and serum ferritin, in health and during infection.
- There is a need for simple instruments that can be used in the field to measure indicators of iron status such as ferritin, transferrin receptor or acute phase proteins, or simple methods to collect samples for analysis, such as dried spots of blood or serum.
- The thresholds and ranges for all indicators of iron status need to be defined and validated for children aged 6–24 months.
- Controlled studies are needed to further examine how body iron stores that have been estimated using the ratio between transferrin receptor and ferritin change in response to interventions to improve iron status.
- Additional iron intervention studies are required to assess the validity of the recommended indicators

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11. List of participants

Dr Pierre Adou, Country Director, Helen Keller International, Nutrition Department, Abidjan, Côte d'Ivoire

Ms Maria Andersson, Technical Officer, Department of Nutrition for Health and Development, World Health Organization, Geneva, Switzerland

Dr Bruno de Benoist, Medical Officer, Department of Nutrition for Health and Development, World Health Organization, Geneva, Switzerland

Professor John Beard, Department of Nutrition Science, Pennsylvania State University, University Park, PA, USA

Dr Jacques Berger, Senior Researcher, Institut de Recherche pour le Développement (IRD), Montpellier, France / Hanoi, Viet Nam

Dr Francesco Branca, Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione, Rome, Italy

Professor Gary Brittenham, Columbia University Medical Center, New York, NY, USA

Professor Chunming Chen, Chinese Center for Disease Control and Prevention, Beijing, China

Dr Christine Clewes, International Micronutritient Malnutrition Prevention and Control (IMMPaCt) Program, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Mary Cogswell, Division of Nutrition and Physical Activity, Centers for Disease Control and Prevention, Atlanta, GA, USA

Professor James Cook, Department of Medicine, University of Kansas, Kansas City, KS, USA

Dr Lena Davidsson (formerly Swiss Federal Institute of Technology), Nutritional & Health-Related Environmental Studies Section, International Atomic Energy Agency, Vienna, Austria

Dr Ines Egli, Technical Officer, Department of Nutrition for Health and Development, World Health Organization, Geneva, Switzerland

Dr Wilma Freire, Chief, Nutrition Unit, WHO Regional Office for the Americas, Pan American Sanitary Bureau, Washington, DC, USA

Dr Laurence Grummer-Strawn, Branch Chief, Maternal and Child Nutrition, Division of Nutrition and Physical Activity, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Andrew Hall, University of Westminster, London, UK (*Rapporteur*)

Dr Eva Hertrampf, Institute of Nutrition and Food Technology (INTA), University of Chile, Santiago, Chile

Professor Lena Hulthén, Institute of Internal Medicine, Sahlgren Academy at Göteborg University, Sahlgren University Hospital, Göteborg, Sweden

Professor Richard Hurrell, Laboratory for Human Nutrition, Institute of Food Science and Nutrition, Swiss Federal Institute of Technology, Zurich, Switzerland

Dr Sa'ad Kharabsheh, Secretary General, Ministry of Health, Amman, Jordan

Professor Prakash Kotecha, Preventive and Social Medicine, Government Medical College, Vadodara, India

Professor Sean Lynch, Department of Clinical Medicine, Eastern Virginia Medical School, Norfolk, VA, USA

Dr Zuguo Mei, Division of Nutrition and Physical Activity, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Nguyen Xuan Ninh, Senior Researcher, Micronutrient Research Department, National Institute of Nutrition, Hanoi, Viet Nam

Professor Omar Obeid, Department of Nutrition and Food Science, American University of Beirut, Beirut, Lebanon

Dr Ibrahim Parvanta, Chief, International Activities, Maternal and Child Nutrition Branch, Division of Nutrition and Physical Activity, Centers for Disease Control and Prevention, Atlanta, GA, USA

Dr Christine Pfeiffer, Chief, Nutrition Laboratory, Division of Laboratory Science (DLS), National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, USA

Professor Rebecca Stoltzfus, Division of Nutritional Sciences, Cornell University, Ithaca, NY, USA (*Chairperson*)

Dr Anna Verster, WHO Senior Technical Advisor, Global Alliance for Improved Nutrition, Geneva, Switzerland

Dr Salvador Villalpando, Instituto Nacional de Salud Publica, Center for Research on Nutrition and Health, Cuernavaca, Mexico

Ms Trudy Wijnhoven, Technical Officer, Department of Nutrition for Health and Development, World Health Organization, Geneva, Switzerland

Professor Pattanee Winichagoon, Deputy Director for Academic Affairs, Institute of Nutrition, Department of Community Nutrition, Mahidol University, Nakhom Pathom, Thailand

Professor Mark Worwood, Department of Haematology, College of Medicine, University of Wales, Cardiff, Wales

Dr Mark Young, Senior Health Adviser, Health Section, United Nations Children's Fund, New York, NY, USA

Appendix 1. The main biochemical indicators of iron status

Measurement	Sample	Commonly used methods[a]	Units	Indicator of	Advantages	Disadvantages
Bone marrow iron (haemosiderin)	Bone marrow aspirate	Microscopical examination of stained marrow cells	Semi-quantitative grading	Depleted or absent body iron stores	Indicates body iron stores and correlates well with other indicators	Invasive and traumatic to collect sample
Haemoglobin	Whole blood	Cyanmethaemoglobin using colorimeter or spectrophotometer or azide-methhaemoglobin using e.g. HemoCue®	g/l	Anaemia	Simple to measure; important functional and public health consequences	Anaemia occurs without iron deficiency; adjustment of thresholds needed for age, sex, pregnancy, altitude, smoking and some ethnic groups
Haematocrit or packed cell volume (PCV)	Whole blood	Centrifugation of whole blood in capillary tube or value derived from automated flow cytometry	Decimal ratio or %	Proportional volume of RBCs in whole blood	Simple to measure	Same as haemoglobin; depends on factors affecting centrifuge e.g. stable power supply
Mean cell volume (MCV)	Whole blood	Calculated from haematocrit and RBC count using haemocyto-meter, or value derived from automated flow cytometry	fl (10^{-12})	Average RBC size: low is microcytic; high is macrocytic	RBC index. Average size of RBCs can be characteristic of type of anaemia	Requires expensive machine to be reliable; low in thalassaemia and inflammation
Mean cell haemoglobin (MCH)	Whole blood	Haemoglobin concentration and RBC count using haemocytometer, or value derived from automated flow cytometry	pg (10^{-9})	Haemoglobin in an average RBC; if low, hypochromic; if normal, normochromic	As for MCV	Requires expensive machine to be reliable; slow to respond to iron deficiency
Red cell distribution width (RDW)	Whole blood	Automated flow cytometry calculates RDW = $\frac{\text{Standard deviation of MCV}}{\text{MCV}}$	%	Abnormal range in size of RBCs <11.5% or >14.5%	Size distribution of RBCs can be characteristic of type of anaemia	Requires expensive machine to be reliable; high in iron deficiency; low in thalassaemia and inflammation
Reticulocyte haemoglobin concentration	Whole blood	Automated flow cytometry	g/l reticulocytes	Concentration of haemoglobin in new RBCs	Represents new RBCs 18-36 hours old, thus recently affected by deficiency	Requires expensive machine to be reliable
Serum or plasma iron	Serum or plasma (not using EDTA)	Colorimetry	µg/dl µmol/l	Iron bound to transferrin in blood	Measure of iron supply to the bone marrow and other tissues	Varies diurnally and after meals; sample easily contaminated with iron from outside sources; low in chronic disease
Erythrocyte protoporphyrin	Whole blood or dried blood spots	Usually estimated from ZPP (below); expressed as ratio to haemoglobin concentration	µg/dl whole blood or RBCs	Restricted supply of iron to developing RBCs	Useful in young children; whole blood or dried spots can be assayed	Increased in iron deficiency, inflammatory disorders, exposure to lead
Zinc protoporphyrin (ZPP)	Whole blood or dried blood spots	Fluorescence spectrophotometry or portable Aviv® haematofluorimeter	µmol/mol of haemoglobin	Lack of iron to developing RBCs	Useful in young children; whole blood or dried spots can be assayed	Increased in iron deficiency, inflammatory disorders, exposure to lead

Measurement	Sample	Commonly used methods[a]	Units	Indicator of	Advantages	Disadvantages
Ferritin	Serum or plasma	Immunoassay e.g. enzyme-linked immunosorbent assay (ELISA) or immunoturbidometry	µg/l	Size of iron stores	Reflects iron status	Ferritin is an acute phase protein so concentration is increased in inflammatory disease and sub-clinical infection
Total iron binding capacity (TIBC)	Serum or plasma	Colorimetric assay of amount of iron that can be bound to unsaturated transferrin *in vitro*; determination from transferrin concentration measured immunologically	µg/dl µmol/l	Total capacity of circulating transferrin bound to iron	Increased in iron deficiency, low in inflammatory disorders	Large overlap between normal values and values in iron deficiency
Transferrin saturation	Serum or plasma	Calculated from: $\frac{\text{Serum iron}}{\text{TIBC}}$	%	Saturation of <15% with high TIBC indicates iron deficiency	Proportion of transferrin bound to iron	Same as for serum iron
Transferrin receptor	Serum or plasma	Immunoassay e.g. ELISA or immunoturbidometry	µg/l	Reflects balance between cellular iron requirements and iron supply	Semi-quantitative measure of the severity of iron deficiency even in presence of inflammatory disorders	Affected by the rate of erythropoiesis
Body iron stores	Serum or plasma	Ratio of transferrin receptor to ferritin $\frac{-[\log(\text{TfR/ferritin ratio}) - 2.8229]}{0.1207}$ [b]	mg/kg	Measure of body iron status including iron deficits, status of storage iron and iron overload	Measure of full range of iron status, validated by phlebotomy studies in adult volunteers	Same limitations as component parts
Hepcidin	Serum or plasma, urine	Immunoassay for pro-hepcidin e.g. ELISA	ng/ml	Regulator of iron absorption from gut	Production diminished when iron reserves depleted	Assay methods and interpretation of results is under development

RBC, red blood cell.

[a] The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization or the Centers for Disease Control and Prevention in preference to others of a similar nature that are not mentioned.

[b] Cook, Flowers, Skikne (*12*).

Appendix 2. Selected biochemical indicators of iron status and an acute phase protein with common methods of measurement, cost, variability, thresholds and reference material

Indicator	Common method or equipment[a]	Approximate cost of equipment US$	Cost/test of supplies and materials US$	Minimum volume for one analysis	Complexity	Sampling/ biological variability[b]	Threshold	Reference material
Haemoglobin	HemoCue® photometer	400	<1	50 µl whole blood	Low	Low/low	110–130 g/l[c]	WHO
Zinc protoporphyrin	Haemato-fluorimetry e.g. AVIV® hemato-fluorimeter	5 000	1	50 µl whole blood	Low	Medium/medium	>70–80 µg/dl red blood cells[c]	Not available
Mean cell volume	Particle counter e.g. Coulter® counter	15 000	5	300 µl whole blood	Low	Low/ low	<67–81 fl[c]	Not available
Transferrin receptor	Immunoassay e.g. ELISA	5 000	10–15	100 µl serum or plasma	Medium	Medium/medium	Not defined[d]	Not available
Ferritin	Immunoassay e.g. ELISA	5 000	5–10	100 µl serum or plasma	Medium	Medium/medium	<12–15 µg/l[c]	WHO
C-reactive protein	Immunoassay e.g. ELISA	5 000	8	50 µl serum or plasma	Medium	Medium/medium	<3–10 mg/l	IFCC[e]
	Orion® QuickRead	3 000	10	20 µl serum or plasma	Low	Medium/medium	<5 mg/l	IFCC[e]

ELISA, enzyme-linked immunosorbent assay.

[a] The mention of specific companies or of certain manufacturers' products does not imply that they are endorsed or recommended by the World Health Organization or the Centers for Disease Control and Prevention in preference to others of a similar nature that are not mentioned.

[b] Sampling variability depends on operator training.

[c] WHO, UNICEF, UNU (*1*).

[d] Use thresholds recommended by manufacturer of assay.

[e] International Federation of Clinical Chemistry.

ANNEX 1

Indicators of the iron status of populations: red blood cell parameters

SEAN LYNCH

Contents

1. Introduction

Iron is an essential nutrient that plays a central role in many metabolic processes. Aerobic metabolism is critically dependent on maintaining normal concentrations of several iron-containing proteins that mediate oxygen transport, storage and utilization, particularly when the tissue demand for oxygen is increased by physical activity. Pioneering research over the last 50 years, much of it stemming from concepts developed and validated experimentally by Dr Clement Finch and his coworkers, led to the recognition that a negative iron balance resulting from an iron intake insufficient to match losses from the body despite compensatory changes in the rate of absorption and, to a more limited extent, excretion could be divided into three stages based on the severity of the potential effect on physiological functions. The evaluation of functional impairment was related entirely to erythropoiesis for two reasons: the effects of changes in iron status on blood elements are readily evaluated, while the effect on the enzymes in other tissues necessitates obtaining biopsy samples. The red blood cell pool is the largest functional iron compartment in the body. Its requirements therefore have a dominant influence on studies of iron transport and storage. The first stage (iron deficiency) is characterized by the absence of measurable iron stores; the second (iron deficient eythropoiesis) by evidence of a restricted iron supply in the absence of anaemia; and the third (iron deficiency anaemia) by a haemoglobin concentration that falls below the normal threshold for age and sex. The iron indicators that can be used to identify the three stages of iron deficiency are discussed in the other literature reviews in this annex.

WHO recognized the public health importance of nutritional anaemia over 50 years ago (*1*) and haemoglobin threshold values to classify anaemia were first published in the report of a 1958 WHO Study Group (*2*). The thresholds were chosen arbitrarily. Revised thresholds were published in 1968 (*3*) based on a review of five earlier reports. The following text dealing with the recommendations is taken from the 1968 report: "The report (*2*) of the 1958 WHO Study Group recommended haemoglobin values below which anaemia could be considered to exist. These figures were chosen arbitrarily and it is still not possible to define normality precisely (*4*). However, more recent data (5–8) indicate that the values given previously should be modified. It is recommended that, in future studies, anaemia should be considered to exist in those whose haemoglobin levels are lower than the figures given below (the values are given in g/100 ml of venous blood of persons residing at sea level):

children aged 6 months to 6 years:	11
children aged 6–14 years:	12
adult males:	13
adult females, non-pregnant:	12
adult females, pregnant:	11 "

Five references were provided by WHO for the more recent data. Four referred to published papers and one to unpublished observations. None of the published references dealt specifically with the development of normal ranges. The first paper (*5*) described a series of observations in 312 healthy Norwegian men, aged 15–21 years. Capillary blood samples were used. A haemoglobin concentration <130 g/l was observed in 3.5% of the sample. The second paper (*6*) was an evaluation of venous blood samples from 149 pregnant women and did not provide any specific recommendations. The third paper (*7*) dealt with a series of experimental observations of venous

blood samples from 82 pregnant women. There were four groups of volunteers: one group served as a control, one received 1000 mg iron intra-muscularly and the remaining two were given a dose of 39 mg oral iron either once or twice a day. The authors suggested that a threshold of 104 g/l should be used to classify anaemia in the last trimester of pregnancy. The fourth paper (*8*) was a report of 600 men aged 35–64 years and 200 women aged 55–64 years in Wales. Venous blood samples were used. The study contained individuals who responded to iron therapy. No specific recommendations for thresholds for anaemia were given.

The WHO thresholds cited above were used by DeMaeyer and Adiels-Tegman (*9*) in their landmark paper which is still often quoted as the basis for estimates of the global prevalence of both all anaemia and iron deficiency anaemia. DeMaeyer and Adiels-Tegman recognized the importance of distinguishing between iron deficiency and other causes of anaemia, and proposed deriving the prevalence of iron deficiency anaemia by subtracting the prevalence of anaemia in men (assuming that the prevalence of nutritional iron deficiency in this group would be negligible in most countries) from the prevalence in other groups, thereby deriving the prevalence of iron deficiency anaemia in these groups. By this means they calculated that generally a little less than 50% of the anaemia could be attributed to iron deficiency. They had no way of estimating the prevalence of iron deficiency without anaemia.

The thresholds proposed by WHO in 1968 are, with minor modifications, still regarded as the international standards for evaluating nutritional iron deficiency anaemia. Moreover their validity has been confirmed by analyses of the US Second National Health and Nutrition Examination Survey after excluding subjects with abnormal tests of iron status. The following thresholds are given in the most recent WHO, UNICEF, UNU guide for programme managers (*10*):

Children 6 months to 59 months	110 g/l
Children 5–11 years	115 g/l
Children 12–14 years	120 g/l
Non-pregnant women (above 15 years of age)	120 g/l
Pregnant women	110 g/l
Men (above 15 years of age)	130 g/l

Most epidemiological surveys in developing countries have focused on women and children. The prevalence of anaemia in men has only been used to evaluate nutritional iron deficiency in a few studies. Iron deficiency anaemia is therefore still generally considered to account for about 50% of the anaemia in surveys that do not include specific measurements of iron status.

2. Relationship between anaemia and iron deficiency

2.1 Physiological control of haemoglobin levels

Haemoglobin concentrations reflect the composite effects of mechanisms that control the sizes of both the red cell mass and the plasma volume. The red cell mass in healthy human beings is maintained by the stimulation of red cell production by the humoral factor erythropoietin. A sensing mechanism within the kidney that is responsive to tissue oxygen concentration controls the release of eythropoietin from renal peritubular cells.

Red cells circulate in the blood for about 90–120 days, necessitating the replacement of about 1% of the red cell mass each day. In normal human beings red cell mass

is controlled by the rate of red cell production, because red cell loss due to senescence is relatively fixed. Red cell mass is increased when oxygen delivery to the blood in the lungs is reduced, such as by high altitude or smoking.

Plasma volume is determined by a complex set of hormonal and vascular factors that control salt and water homeostasis, blood pressure and vascular permeability.

The range of normal haemoglobin concentrations used to evaluate individuals for clinical diagnostic purposes is customarily determined from the distribution of haemoglobin concentrations in healthy population groups. An arbitrary proportion of the healthy population (usually 2.5%) is assumed to fall below the appropriate threshold. The variation in haemoglobin values in healthy human beings is relatively large; in women aged 18–49 years the mean haemoglobin concentration is 140 g/l while the value 2 standard deviations (SD) below the mean is 120 g/l, a difference of about 14%. Table 1 gives the normal mean values and lower limits for a Caucasian population published in a popular current textbook of haematology.

TABLE 1
The mean and lower standard deviation (–2 SD) of normal haemoglobin concentrations (g/l) in a Caucasian population

Age range	Mean	-2 SD
3–6 months	115	95
0.5–2 years	120	110
2–6 years	125	115
6–12 years	135	115
12–18 years females	140	120
12–18 years males	145	130
18–49 years females	140	120
18–49 years males	155	135

Adapted, from Hoffman (*11*), with permission of the publisher.

2.2 Relationship between iron deficiency and anaemia

Individuals with iron deficiency anaemia are a subset of all the anaemic individuals in a population. They can be identified as iron deficient by using measurements of laboratory indicator(s) that are more specifically influenced by iron status than the haemoglobin concentration. However the proportion of low haemoglobin values is often used alone to predict the prevalence of iron deficiency anaemia. This approach is flawed for several reasons.

There is a significant overlap in the distribution of haemoglobin values of iron sufficient individuals and those with true iron deficiency anaemia, meaning anaemia that responds to treatment with iron. Garby et al. (*12*) demonstrated this by showing an overlap in the distribution of haematocrit values between iron sufficient and iron deficient individuals (determined by their responses to treatment with iron) in a group of apparently healthy women of childbearing age from Uppsala in Sweden. In this population a woman with a haemoglobin concentration at the WHO threshold of 120 g/l (equivalent to a haematocrit of 36%) would have a probability of only 58.5% of being iron deficient (*13*). Margolis et al. (*14*) reported similar results for children. Two other approaches have been used to demonstrate the overlap in the distributions of haemoglobin values between iron deficient and iron sufficient samples of people. Cook et al. (*15*) estimated the prevalence of anaemia in pregnant and non-pregnant

women in Latin America by resolving the distribution of haemoglobin values in the sample into two Gaussian distributions. Using the WHO threshold for pregnant women of 110 g/l, 23% of the women with anaemia were wrongly classified as normal while 27% of the normal population were misclassified as suffering from anaemia. Anaemia defined by the current WHO criteria was present in 38.5% of pregnant and 17.3% of non-pregnant women. The corresponding values based on an analysis of the frequency distribution were 22% and 12% respectively. Cohen et al. (*16*) used a similar distribution analysis to identify iron deficiency anaemia in children aged 12–23 months in Guatemala. Dallman et al. (*17*) employed a "median shift" method to estimate the prevalence of iron deficiency anaemia in the United States of America. All of these examples demonstrate the significant flaw arising from the assumption that individuals with iron deficiency anaemia can be readily identified by applying simple thresholds to the distribution of haemoglobin concentrations.

The value of using haemoglobin measurements to define the prevalence of iron deficiency in healthy population groups is further diminished by the need to make adjustments to the thresholds for individuals who live at high altitudes or smoke regularly (*18*). There is also convincing evidence that there are small differences in the distributions of haemoglobin values between different ethnic groups. Suggested adjustments to thresholds for the ethnic groups that have been studied are given in Table 2. It is important to note that differences in the distribution of haemoglobin concentrations have only been established for a few ethnic groups.

TABLE 2
Suggested adjustments for several ethnic groups to the thresholds of haemoglobin concentration used by WHO to define anaemia

Ethnic Group	Suggested adjustment (g/l)	References
African Americans	-10.0	(*18*)
East Asian Americans	0	(*18*)
Hispanic Americans	0	(*18*)
Japanese Americans	0	(*18*)
American Indians	0	(*18*)
Jamaican girls (13–14 yrs)	-10.7	(*19*)
Indonesians from West Indonesia	0	(*20*)
Thais	0	(*21*)
Vietnamese	-10.0	(*22*)
Greenland men Greenland women	-8.0 -6.0	(*23*)

Several other factors limit the value of anaemia as a means to diagnose iron deficiency. Iron deficiency is not the only cause of anaemia and in some circumstances it may not be the most common cause. Vitamin A deficiency is probably the second most frequent nutritional cause of mild anaemia, while deficiencies of folic acid, vitamin B_{12} and possibly riboflavin may also cause anaemia. Inflammatory and infectious diseases including malaria are undoubtedly as common as iron deficiency as a cause of anaemia in many developing countries. Anaemia may also result from inherited or acquired conditions that affect red cell production. People who are carriers of alpha- or beta-thalassaemia are the most likely to be included in surveys. Both carrier states may cause a mild microcytic anaemia. Finally, there may be seasonal variations in haemoglobin concentration: variation was reported in a cross-sectional

study of men in Israel (*24*,*25*). Small variations may also occur during the menstrual cycle with mean values 3 g/l lower during menses than during the luteal phase (*26*).

The prevalence of iron deficiency without anaemia can only be inferred if specific iron indicators are not included in epidemiological surveys. Iron deficiency may have functional consequences. An effect of iron deficiency without anaemia on the cognitive and motor development of children has not been established although there is considerable experimental evidence to support the contention that the functional consequences result from tissue iron deficiency within the brain rather than from the accompanying anaemia (*27*). Physical performance is known to be affected by iron deficiency in the absence of anaemia. The maximum oxygen consumption was reduced in iron deficient women without anaemia when compared with an iron sufficient control group (*28*). Aerobic adaptation was improved by the administration of iron supplements to trained and untrained women who were not anaemic (*29*,*30*). Although functional consequences in the absence of anaemia may not have been established for the effects of iron deficiency other than on physical performance, it is important to emphasize again the difficulty of identifying anaemia in some individuals because of the overlap in the distribution of haemoglobin concentrations between iron deficient and iron sufficient people. Furthermore individuals whose iron intake is marginal may or may not be anaemic at different times because of small changes in iron intake or bioavailability.

3. Red blood cell parameters

3.1 Haemoglobin

The measurement of haemoglobin concentration and other red blood cell parameters is well standardized. Laboratories that use particle counting and sizing equipment have access to expert technical support and quality control procedures. However, practical considerations will limit the use of such equipment, particularly during field studies in remote regions, because it may be difficult to take blood samples to a laboratory on the day of collection. It is therefore appropriate to focus on the use of instruments such as the Hemocue (Hemocue AB, Ängelholm, Sweden). It is possible to get highly reproducible results when the Hemocue is used to measure the haemoglobin concentrations of venous blood samples, but there may be considerable variability when the Hemocue is used to measure the haemoglobin concentrations of capillary blood samples. This variability is caused by poor capillary blood sampling techniques. The appropriate use of the Hemocue and capillary sampling techniques is currently a very important issue that is dealt with in a recent manual published by Helen Keller International (*31*). A manual from the International Nutritional Anemia Consultative Group on "Measurements of Iron Status" (*32*) also provides useful information.

3.2 Hematocrit or packed cell volume

The measurement of hematocrit does not supply any information about anaemia that cannot be obtained from measuring the haemoglobin concentration. The hematocrit is a derived value on particle counters. Values measured directly by centrifuging blood samples in the field tend to have poor reproducibility unless a stable power supply can be assured and the equipment is well standardized.

3.3 Mean cell volume and mean cell haemoglobin

Mean cell volume (MCV) and mean cell haemoglobin (MCH) provide identical information and are interchangeable in terms of their value in identifying nutritional iron deficiency. MCV is the value that has been used most widely for the evaluation of nutritional iron deficiency.

A low MCV is not specific to iron deficiency. Low values are encountered in thalassaemia (2 or 3 gene deletions for alpha thalassaemia, beta thalassaemia including heterozygotes) and in about 50% of people with anaemia due to inflammation.

Reliable measurements of MCV require a particle counter and are therefore unlikely to provide an advantage over specific measurements of iron status in terms of cost and technical support.

3.4 Red cell distribution width

The initial enthusiasm for the use of red cell distribution width (RDW) to distinguish between iron deficiency anaemia and the anaemia of inflammatory disorders has not been supported by subsequent studies (*33*). Measurements of RDW are made using a particle counter.

3.5 Reticulocyte haemoglobin concentration and percentage of hypochromic red cells

Reticulocyte haemoglobin concentration has been shown to be a reliable method for identifying iron deficiency and has been recommended for the evaluation of the adequacy of the iron supply in patients undergoing dialysis for renal failure who are receiving erythropoietin (*34–38*). The measurement has been compared to the "gold standard" for iron deficiency, which is stainable bone marrow iron, and found to be reliable. While it promises to be a useful tool for evaluating iron supply even in clinically complex settings, it currently requires access to a specific brand of flow cytometer. It is therefore unlikely to be helpful in developing countries. Moreover reticulocyte haemoglobin concentrations seem to be unsuitable for evaluating iron status in alpha and beta thalassaemias, including carrier states.

4. Role of haemoglobin as a screening indicator for iron deficiency

It is generally assumed that worldwide "at least half the anaemia is due to nutritional iron deficiency" (*39*) and that "up to a prevalence of iron deficiency anaemia of 40%, the prevalence of iron deficiency will be about 2.5 times that of anaemia" (*10*). However, it is evident from the foregoing discussion that there are considerable variations in both of these ratios depending on the age and sex of the people being studied, the region of the world in which they live, and the prevalence rates of other causes of anaemia. The prevalence of anaemia alone can therefore give only a very rough estimate of the likely prevalence of iron deficiency anaemia. Attempts to analyse the variation in the relationship between prevalence rates for iron deficiency and anaemia are confounded by the absence of a consistent standard for identifying iron deficiency. There are very few studies that have used bone marrow iron, which is clearly too invasive an indicator to be applied routinely in field studies. Various combinations of iron indicators have been used, but the thresholds for iron deficiency vary.

For example: the threshold at which ferritin indicates iron deficiency may be set as high as 30 µg/l in some developing countries because of the presence of infectious diseases (*40*,*41*); thresholds for zinc protoporphyrin vary between 40 and 70 µmol/mol haem depending on whether the cells have been washed before the assay or not (*42*,*43*); and there is a lack of standardization between different commercial kits for measuring the concentration of transferrin receptor.

The response of the haemoglobin concentration to treatment has long been the primary indicator for evaluating the efficacy or effectiveness of intervention programs. It remains a satisfactory indicator for this purpose, but lacks sensitivity when the prevalence of anaemia is low. Furthermore, if there is a significant residual prevalence of anaemia at the end of the intervention (perhaps despite a considerable reduction when compared with the baseline values) it may be impossible to determine whether the intervention was suboptimal or not as the residual anaemia may be unrelated to iron deficiency. Despite the drawbacks outlined above it has been suggested that the prevalence of anaemia may be the best epidemiological indicator of iron status in infants and young children because of the poor performance of the other currently available indicators (*14*).

5. Conclusions

1. Anaemia is an inevitable consequence of iron deficiency of sufficient severity.
2. Nutritional iron deficiency anaemia is a mild anaemia because iron loss is reduced to some extent in iron deficiency (*44*).
3. A significant overlap in the distributions of haemoglobin concentration of iron deficient and iron sufficient individuals has been demonstrated in children, non-pregnant women, and during pregnancy.
4. A low haemoglobin concentration is not a specific indicator of iron deficiency anaemia. Other epidemiologically important causative factors include: infectious and inflammatory disorders, the prevalence of genes for thalassaemia, vitamin A deficiency, and deficiencies of folic acid and vitamin B_{12} in certain settings.
5. The current WHO thresholds provide the best separation between iron deficient and iron sufficient individuals in a population. They should be adjusted for altitude, smoking and, where information is available, ethnic origin.
6. Haemoglobin concentration can be used as an initial screening indicator. However, because of variations in the relationships between iron deficiency, iron deficiency anaemia and anaemia from other causes, the prevalence of iron deficiency cannot be predicted with any degree of accuracy. Further evaluation using specific iron indicators is necessary. It may be possible to agree on an anaemia prevalence below which further evaluation for iron deficiency would not be warranted in certain age or sex groups.
7. The change in the haemoglobin concentration and the prevalence of anaemia can be used to provide a qualitative or a semi-quantitative assessment of the efficacy of intervention strategies, but the residual prevalence of iron deficiency cannot be predicted.
8. Anaemia is a useful clinical and experimental criterion for defining the severity of iron deficiency. It may be less useful in epidemiological surveys. Consideration should be given to developing a new set of criteria, based on specific laboratory indicators of iron status, for defining the severity of iron deficiency in epidemiological surveys.

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ANNEX 2

Indicators of the iron status of populations: ferritin

MARK WORWOOD

Contents

1. Sources of data

This review summarises the genetics, biochemistry and physiology of serum ferritin, discusses variables affecting the assay of ferritin, and examines how ferritin may be used to assess the iron status of populations. It builds on a review of earlier studies of serum ferritin concentration in health and disease by Worwood (*1*) and on several national and international reviews of using serum ferritin to determine iron status (*2–4*). The Centers for Disease Control and Prevention Nutrition Laboratory has recently prepared a *Reference Manual for Laboratory Considerations – Iron Status Indicators for Population Assessments* (2003), which covers all aspects of the processing of blood samples.

2. Introduction

The iron storage protein ferritin is found in both prokaryotes and eukaryotes. It consists of a protein shell with a molecular mass of about 500 kDa composed of 24 subunits. The protein shell encloses a core of ferric-hydroxy-phosphate which can hold up to 4 000 atoms of iron. Proteins with a similar overall structure are found throughout the plant and animal kingdom as well as in bacteria, although bacterial ferritin appears to have evolved separately as it has no amino acid sequence homology with animal ferritins. Bacterial ferritin from *Escherichia coli* for example, contains haem (about one per two subunits), as well as a core of non-haem iron. Ferritin is ancient in evolutionary terms and also has a long biochemical history. Since it was first isolated (*5*) two main issues have dominated ferritin research: its structure, and the mechanism of iron uptake and release. Recently the molecular biology of ferritin has come to the fore and the molecule has become a model for studies of how synthesis is regulated at the level of genetic translation. A detailed review of the structure and function of ferritin has been published (*6*).

3. Genetics

A range of isoferritins is found in various human tissues. These are composed of combinations of two types of subunit, H and L (*7*). The expressed gene for the H-subunit is on chromosome 11 at 11q13 (*8*) and that for the L-subunit is on chromosome 19 at 19q13-ter. There are however multiple copies of the ferritin genes. Most of the H sequences (about 15 copies) on a number of chromosomes appear to be processed pseudogenes (i.e. without introns) with no evidence for their expression. The same applies to the other 'L' sequences found on chromosomes 19, 21 and X. However an intronless gene on chromosome 5q23.1 codes for mitochondrial ferritin – a newly identified H-type ferritin (*9*). For the expressed L-gene in the rat there are three introns located between exons coding for the four major α–helical regions of the peptide sequences. Human H and L genes have a similar structure although the introns differ in size and sequence. The messenger ribonucleic acid (mRNA) for the human ferritin genes contains about 1.1 kb. The H-subunit is slightly larger than the L-subunit (178 amino acids compared with 174 amino acids) but on electrophoresis in polyacrylamide gels under denaturing conditions the apparent differences in relative molecular mass are rather greater (21 kDa and 19 kDa). Human H and L sequences are only 55% homologous whereas the degree of homology between L-subunits and H-subunits from different species is of the order of 85% (*6*).

4. Structure

A ferritin subunit has five helices and a long inter-helical loop. The loop L and the N-terminal residues are on the outside of the assembled molecule of 24 subunits. The C-terminal residues are within the shell. H and L chains adopt the same conformation within the molecule. A description of the three-dimensional structure of apoferritin will be found in a recent review (*6*).

In human tissues H-rich isoferritins (isoelectric point (pI) 4.5–5.0) are found in heart muscle, red blood cells, lymphocytes, monocytes, HeLa cells and other, but not all, cultured cells (*10*). L-rich isoferritins are more basic (pI 5.0–5.7) than H-rich isoferritins and are found in the liver, spleen and placenta. The pI of ferritin is not significantly affected by its iron content, which varies from tissue to tissue and with the tissue iron content.

Ferritin is purified from tissues by taking advantage of three properties: the ability to withstand a temperature of 75 °C; the high density of the iron-rich molecule, which allows concentration by ultracentrifugation; and crystallisation in the presence of cadmium sulphate. However it should be noted that, whereas ultracentrifugation tends to concentrate molecules rich in H-subunits, crystallisation from cadmium sulphate solution tends to give a lower overall recovery and selects molecules rich in L-subunits (*11*).

5. Haemosiderin

Ferritin is a soluble protein but is degraded to insoluble haemosiderin which accumulates in lysosomes. Both ferritin and haemosiderin provide a store of iron that is available for protein and haem synthesis. Normally much of the stored iron in the body (about 1 g in men and less in pre-menstrual women and children) is present as ferritin, but during iron overload the proportion present as haemosiderin increases. Purified preparations of ferritin always contain a small proportion of molecules in the form of dimers, trimers and other oligomers (*12*). These may be intermediates in the formation of haemosiderin. Andrews et al. (*13*) isolated a soluble ferritin from iron-loaded rat liver lysosomes which contained a peptide of molecular mass 17.3 kDa which may be a precursor of insoluble haemosiderin. Peptides extracted from preparations of haemosiderin have been found to react with antibodies to ferritin (*14*,*15*).

6. Regulation of ferritin synthesis and breakdown

Ferritin synthesis is induced by administering iron. In 1966 Drysdale and Munro (*16*) showed that the initial response of apoferritin synthesis to the administration of iron was by regulating translation rather than transcription. This requires the movement of stored mRNA from the ribonucleoprotein fraction (RNP) to the polysomes (*17*) followed by an increased rate of translation of ferritin subunits. This response is the same for H and L subunits. However, after administering iron there is an eventual increase in the rate of transcription of the L-subunit gene. This causes an increase in the ratio of L- to H-subunits during ferritin synthesis after administering iron (*18*). The translational control mechanism involves the 5' untranslated region of the ferritin mRNA which contains a sequence forming a 'stem-loop' structure. This is called an 'iron response element' (IRE). Similar cytoplasmic proteins (IRP- and -2)

bind to the IRE in the absence of iron but are inactivated (IRP-1) or degraded (IRP-2) when iron supply increases (*19*). Binding to mRNA prevents ferritin synthesis, but in the absence of binding, polysomes form and translation proceeds. The protein IRP-1 is the iron-sulphur protein, aconitase, encoded by a gene on chromosome 9 which functions as a cytosolic aconitase in its iron-replete state. A model involving conformation changes which permit RNA binding has been proposed (*20*). A related mechanism operates in reverse for the transferrin receptor. Here there are stem-loop sequences in the 3' untranslated region, and protein binding prevents degradation of mRNA. Hence iron deficiency enhances transferrin receptor synthesis. Erythroid ALA synthase, aconitase, DMT1 and ferroportin-1 also have IREs.

Although ferritin is generally considered to be an intra-cellular protein and most of the mRNA in the liver is associated with free ribosomes, there is evidence of the synthesis of ferritin on membrane-bound polysomes (21). This finding may be of special relevance to the origin of plasma ferritin (see below).

The way in which ferritin is degraded remains largely a mystery. Studies of rat liver cells (16) indicate that the half-life of a ferritin molecule is about 72 hours, and is extended by iron administration. The relationship between ferritin breakdown and formation of haemosiderin is unclear, as is the fate of the iron core after the degradation of the protein shell.

7. Functions related to iron storage

The major function of ferritin is clearly to provide a store of iron which may be used for haem synthesis when required. Iron uptake *in vitro* requires an oxidizing agent, and iron release requires a reducing agent (reviewed by Harrison and Arosio (*6*). There are differences in the rate of iron uptake between apoferritins with varying proportions of H and L-subunits; H-rich isoferritins having the highest rate of iron uptake *in vitro* (*22*). Such isoferritins are found in cells which either have a high requirement for iron for haem synthesis, such as nucleated red cells and cardiac muscle, or which do not appear to be involved in iron storage, such as lymphocytes. In the tissues where iron is stored, such as the liver and spleen, the ferritin contains mostly L-subunits. Recent studies with recombinant H_{24} and L_{24} molecules have demonstrated that the ferroxidase activity of ferritin is a property of the H-subunit and that L24 molecules have little ability to catalyse iron uptake (*6*). The maturation of monocytes to macrophages *in vitro* is associated with the loss of acidic isoferritins (*23*). Iron storage therefore seems to require ferritin that is rich in L-subunits.

8. Are there tumour specific ferritins?

There has been considerable interest in specific 'carcino-fetal' ferritins , which are molecules peculiar to fetal or malignant cells. This term originated in a paper by Alpert et al. (*24*) to describe the acidic ferritins found in rat fetal liver cells and in some neoplastic tissues. It is now accepted that the variation in isoelectric point of ferritin molecules from various tissues is effectively explained in terms of the two-subunit model of Arosio et al. (*7*). Later, Moroz et al. (*25*) chose human placental ferritin as a possible source of unique, antigenic "onco-fetal" ferritin, and produced a monoclonal antibody (H9) which bound to placental ferritin but not to liver or spleen ferritin. Recently the unique subunit in placental ferritin has been fully characterised (*26*) and named placental immunomodulatory ferritin (PLIF). The PLIF coding region is

composed of ferritin heavy chain (FTH) sequence lacking the 65 C-terminal amino acids, which are substituted with a novel 48 amino acid domain (C48). In contrast to FTH, PLIF mRNA does not include the iron response element in the 5'-untranslated region, suggesting that PLIF synthesis is not regulated by iron. The authors suggest that the p43 subunit of PLF may represent a dimer of PLIF (22 kDa). Furthermore, PLIF transcripts exist at a very low copy number compared with ferritin heavy chain in placental tissues at term delivery (40 weeks). Despite the development of an immunoassay for placental isoferritin (*27*) the assay has not been widely exploited in the diagnosis of cancer and its specificity for malignancy has not been established. Low concentrations of placental ferritin in serum during pregnancy may indicate abnormal gestation (*28*).

9. Ferritin as a regulator of erythropoiesis

A role for ferritin in the regulation of haemopoiesis, apparently unrelated to iron storage, was proposed by Broxmeyer et al. (*29*) who showed that the protein responsible for a 'leukaemia-associated inhibitory activity' (LIA) was an acidic isoferritin. This protein fraction, and an acidic isoferritin preparation from the spleen of a patient with chronic myeloid leukaemia, suppressed colony formation *in vitro* of CFU-GM, BFU-E and CFU-GEMM progenitor cells from the marrows of normal donors, but was ineffective in marrow or blood from patients with acute leukaemia, myelodysplasia and some other haematological disorders.

Ferritin appears to act *in vitro* on progenitors which are in the DNA synthesis (S) phase of the cell cycle (*30*). Sala et al. (*31*) were unable to confirm the original results of Broxmeyer et al. (*29*) and since 1992 there has been little work on this inhibitory activity of ferritin.

10. Plasma (serum) ferritin

It was only after the development of a sensitive immunoradiometric assay (IRMA) that ferritin was detected in the serum or plasma of normal individuals (*32*). Reliable assays, both radioimmunoassay (RIA) using labelled ferritin and IRMA using labelled antibody, have been described in detail (*33*). These assays have since been supplanted by enzyme linked immunoassays (ELISA) using colorimetric and fluorescent substrates or by antibodies with chemiluminescent labels. The solid phase may be a tube, bead, microtitre plate or magnetic particle. Numerous variations have been described and serum ferritin is included in the latest batch and random access, automated analysers for immunoassays.

10.1 Relationship to storage iron levels

Serum ferritin concentrations are normally within the range 15–300 µg/l and are lower in children than adults (Table 1 and 2). Mean values are lower in women before the menopause than in men, reflecting women's lower iron stores caused by the losses during menstruation and childbirth. The changes in serum ferritin concentration during development from birth to old age reflect changes in the amounts of iron stored in tissues (*34*). A mother's iron status appears to have relatively little influence on the concentration in cord serum, and mean values are in the range 100–200 µg/l. There is a good correlation between serum ferritin concentration and storage iron

TABLE 1

The normal range in mean serum ferritin concentration (µg/l) of adults by sex and age group[a]

	Men				Women			
Age range (years)	Sample size	Mean serum ferritin concentration (µg/l)	5th percentile	95th percentile	Sample size	Mean serum ferritin concentration (µg/l)	5th percentile	95th percentile
18–24	107	80	15	223	96	30	5	73
25–34	211	108	21	291	226	38	5	95
35–44	202	120	21	328	221	38	5	108
45–54	166	139	21	395	177	60	5	217
55–64	140	143	22	349	162	74	12	199
65–74	127	140	12	374	138	91	7	321
75+	80	110	10	309	99	77	6	209
Total	1033	121	16	328	1119	56	5	170

[a] Subjects being treated with drugs for iron deficiency (n = 26) were included.
Adapted from White et al. (*40*), with permission of the publisher.
For other surveys of populations in North America and Europe, see Cook et al. (*42*), Finch et al. (*43*), Jacobs and Worwood (*44*), Milman et al. (*45*), Valberg et al. (*46*), Custer et al. (*47*).

TABLE 2

The mean and normal range in the serum ferritin concentration (µg/l) of infants, children and adolescents

Number of children	Age	Population	Selection	Mean serum ferritin concentration (µg/l)	Range serum ferritin concentration (µg/l)	Reference
46	0.5 months	Helsinki	Non-anaemic	238	90–628	Saarinen and Siimes (*48*)
46	1 month	Helsinki	Non-anaemic	240	144–399	Saarinen and Siimes (*48*)
47	2 months	Helsinki	Non-anaemic	194	87–430	Saarinen and Siimes (*48*)
40	4 months	Helsinki	Non-anaemic	91	37–223	Saarinen and Siimes (*48*)
514	0.5–15 years[a]	San Francisco	Non-anaemic	30[b]	7–142	Siimes et al. (*49*)
323	5–11 years	Washington	Low income families	21[b]	10–45[c]	Cook et al. (*42*)
117	5–9 years	Nutrition Canada Survey	Random	15[d]	2–107[e]	Valberg et al. (*46*)
335	6–11 years	Denmark	Random, urban	29[a]	12–67[f]	Milman and Ibsen (*50*)
126 male 125 female	12–18 years	Washington	Low income families	23[b] 21[b]	10–63[c] 6–485	Cook et al. (*42*)
98 male 106 female	10–19 years	Nutrition Canada Survey	Random	18[d] 17[d]	3–125[e] 2–116[e]	Valberg et al. (*46*)
269 male 305 female	12–17 years	Denmark	Random, urban	28[b] 25[b]	11-68[f] 6–65[f]	Milman and Ibsen (*50*)

[a] There were no significant differences in median values for ages 6–11 months, 1–2, 2–3, 4–7, 8–10 and 11–15 years
[b] Median
[c] 10–90 percentile
[d] Geometric mean
[e] Confidence interval
[f] 5–95% interval

mobilized as a result of phlebotomy. This suggests a close relationship between the total amount of stored iron and the serum ferritin concentration in normal individuals (*35*). Serum ferritin concentration decreases with blood donation (*36–38*) and increases with alcohol intake (*36,39,40*). The significant association with alcohol consumption in both men and women has been confirmed in the Health Survey for England (40). In this survey the ferritin concentration was also higher with increasing body mass index. In women after the menopause the ferritin concentration increases but remains lower than in men (Table 1 and 6). In unselected elderly patients a high concentration of ferritin is often associated with disease (*41*). The serum ferritin concentration is relatively stable in healthy persons (see below). In patients with iron deficiency anaemia, the serum ferritin concentration is typically less than 12–15 µg/l. This threshold has been established in a number of studies by determining the serum ferritin concentrations of patients with iron deficiency anaemia (see below) and a reduction in the level of reticuloendothelial iron stores is the only, common, cause of a low serum ferritin concentration. This is the key to the use of the serum ferritin assay in clinical practice (*34*). A high concentration of serum ferritin is found during iron overload, but there are other causes as well.

10.2 Serum ferritin in acute and chronic disease

The acute phase refers to a series of events that occur in response to infection or tissue damage. The local reaction is termed inflammation and the systemic response is referred to as the acute phase response. The acute phase response may be induced by toxic chemicals, physical trauma, infection, inflammation, malignancy, tissue necrosis (e.g. myocardial infarction) and immunisation. The clinical and metabolic features of the acute phase response include fever, leucocytosis, thrombocytosis and metabolic alterations, as well as changes in the concentration of a number of plasma proteins. The changes in several plasma proteins including ferritin during infection, inflammation and trauma are discussed in the review by Northrop-Clewes.

In the anaemia of chronic disease the most important factor controlling serum ferritin concentration is the level of storage iron. However the serum ferritin concentration is higher than in patients with similar levels of storage iron but without infection and inflammation. There is experimental evidence from studies of rat liver cells that the rapid drop in serum iron concentration which follows the induction of inflammation may be due to an increase in apoferritin synthesis which inhibits the release of iron to the plasma (*51*). Interleukin-1 (IL-1) is the primary mediator of the acute-phase response which, in iron metabolism, is indicated by a drop in plasma iron concentration (*52*). There is direct evidence from studies of cultured human hepatoma cells that IL-1ß (which also causes changes in protein synthesis which mimic the acute phase response in cultured hepatoma cells) directly enhances the rate of ferritin synthesis by control of translation (*53*).

Few longitudinal studies of serum ferritin have been reported. After experimentally inducing fever in normal volunteers, ferritin concentrations reached a maximum after 3 days and gradually returned to normal values over the next 10 days (*54*). The increases were relatively small, with ferritin concentrations increasing by about 20 µg/l per 24 hour after giving etiocholanolone. After acute infection, there were increases of about 3 fold in serum ferritin concentration, with the maximum concentration reached within 1 week (55). Concentrations then declined slowly over several weeks. After myocardial infarction, ferritin concentrations began to rise after 30

hours, reached a peak after about 4 days, and then remained above initial levels for up to two weeks (*55,56*). The increases in serum ferritin were smaller than those found for acute infection. After surgery there was a rapid decline (in 2 days) in the concentration of haemoglobin, serum iron and transferrin, with the greatest fall shown by serum iron (*57*). The concentration of C-reactive protein (CRP) rose less rapidly from <5 mg/l to over 100 mg/l, reaching a maximum value at 5 days, and then declined to normal values over the subsequent 4 weeks. Most of the decrease was in the first 10 days. Ferritin increased to a maximum concentration at about 5 days, but the mean increase was less than 50%. After 4 weeks values returned to those before surgery. Serum transferrin receptor (sTfR) concentrations showed little change after surgery.

The combined effect of these changes on blood viscosity and erythrocyte aggregation may be detected by directly measuring plasma viscosity or the erythrocyte sedimentation rate (ESR). Changes occur slowly and these measures are of greater use in monitoring chronic disease than in detecting the immediate response to injury.

The Expert Panel on Blood Rheology of the International Committee for Standardization in Haematology (ICSH), has published guidelines on measuring the ESR and blood viscosity (*58*). Suitable methods for determining the CRP concentration include nepholometry and turbidimetry, and they should be able to detect the protein at concentration as low as 5 mg/l. An international reference standard is available (*59*).

Many clinical studies have demonstrated that patients with anaemia of chronic disease and no stainable iron in the bone marrow may have a serum ferritin concentration considerably in excess of 15 µg/l and there has been much debate about the practical application of the serum ferritin assay in this situation (*60*). A ferritin concentration of <15 µg/l indicates the absence of storage iron while concentrations >100 µg/l indicate the presence of storage iron. Concentrations in the range of 15–100 µg/l serum ferritin are difficult to interpret. It would seem logical to combine the assay of serum ferritin with a measure of disease severity such as the ESR or the concentration of CRP. Witte et al. (*61*) described such an approach and claimed to be able to confirm or exclude iron deficiency, defined as an absence of stainable iron in the bone marrow, in almost all patients with secondary anaemia. However these findings have not been confirmed (*62*). This lack of success in "correcting" serum ferritin concentrations for the effect of inflammation or infection is probably due to the different responses to acute disease shown by ferritin and CRP. Although other acute phase proteins may show similar responses in time, the small changes in concentration reduce the value of the marker as an indicator of disease. Minor infections in children, without changes in other markers of infection, may cause long-term increases in serum ferritin concentration (*63*). Minimal inflammation, detected using a highly sensitive assay for CRP, led to a low serum iron concentration in infants (*64*). As described earlier, measurements of soluble transferrin receptor concentration may provide a valuable diagnostic aid for this difficult area of nutritional assessment.

In assessing of the adequacy of iron stores to replenish haemoglobin, the degree of anaemia must also be considered. Thus a patient with a haemoglobin concentration of 100 g/l may benefit from iron therapy if the serum ferritin concentration is below 100 µg/l (*65*). This is discussed below in terms of the predictive power of diagnostic tests.

10.3 Serum ferritin and liver disease

The other major influence confounding the use of the serum ferritin concentration to estimate iron stores is liver disease. The liver contains much of the iron stored in the body, and any process that damage liver cells will release ferritin. It is also possible that liver damage may interfere with clearance of ferritin from the circulation. It was suggested by Prieto et al. (*66*) that the ratio of serum ferritin to aspartate aminotransferase activity might provide a good index of liver iron concentration. Glycosylated ferritin concentrations might be related directly to storage iron concentrations, while the concentration of non-glycosylated ferritin would relate to the degree of liver damage (*67*). However neither the ferritin:aspartate aminotransferase ratio (*68*,*69*) nor the measurement of glycosylated ferritin concentration (33,70) have proved to be any more reliable than the simple measurement of serum ferritin concentration as an index of liver iron concentration. In patients with liver damage a low serum ferritin concentration always indicates absent iron stores, a normal concentration indicates absent or normal iron stores but rules out iron overload, whereas a high concentration may indicate either normal or high iron stores and further investigation may be necessary to distinguish between the two.

10.4 Serum ferritin concentration and malignancy (*34*)

A high concentration of ferritin is seen in most patients with pancreatic carcinoma, lung cancer, hepatoma and neuroblastoma, although in most cases of cancer of the oesophagus, stomach and colon, the serum ferritin concentration is within the normal range. In breast cancer, the concentration is usually raised in patients with metastatic disease, but the assay has not proved to be useful in predicting metastasis. Patients with acute leukaemia generally have a higher serum ferritin concentration than normal but this is not the case for patients with chronic leukaemia. In Hodgkin's disease the concentration of ferritin increases with the stage of disease, but is not related to the histological type of disease.

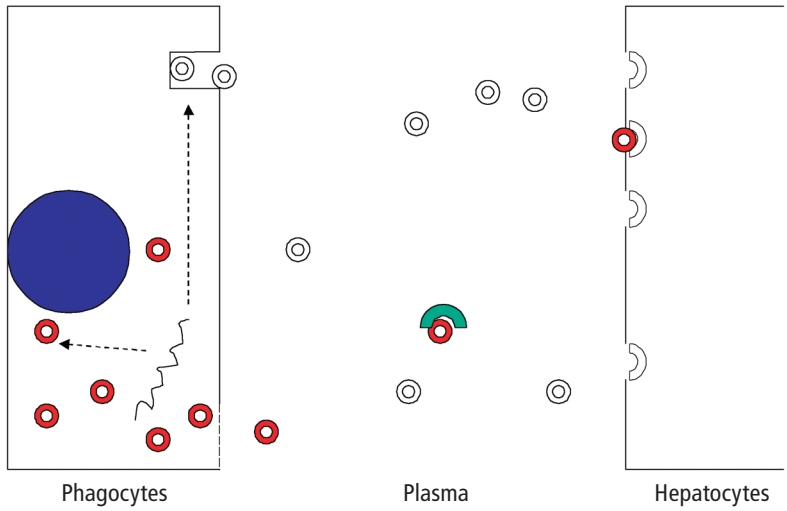
The concept of carcino-fetal ferritin has been introduced above and a logical extension of the concept is to search for changes in the immunological properties of serum ferritin in order to detect malignant disease or monitor the effect of therapy. A number of assays have been described using acidic isoferritins derived from HeLa cells (*71–73*) or heart ferritin (*74*,*75*) and have been applied to serum from patients with cancer. The results have been inconsistent, but later studies using a monoclonal antibody (*76*) confirm some of the studies with polyclonal antisera and indicate that the concentration of H-rich isoferritin in serum is very low compared with L-rich isoferritins, even in patients with cancer. An assay for placental isoferritin has not improved tumour specificity (see above). It is likely that the high concentration of ferritin in the serum in malignancy is due to an increase in the concentration of storage iron, to liver damage, or to inflammation, as well as a consequence of the direct release of ferritin from the tumour. Whatever the cause is, the result is an increase in the concentration of L-rich isoferrritin in the serum rather than accumulation of 'tumour-specific' isoferritins.

10.5 Exceedingly high serum ferritin concentrations

The factors controlling plasma ferritin concentration are: 1) synthesis, 2) release from cells), 3) clearance from the plasma (Figure 1). There are no instances yet known in which a very high ferritin concentration is due to abnormalities in ferritin clearance, but abnormalities occur in both synthesis and release.

In iron overload the serum ferritin concentration is unlikely to exceed 4 000 µg/l in the absence of concomitant liver damage (*33*) but in liver necrosis the ferritin concentration may be in excess of 50 000 µg/l (*66*). The stimulation of synthesis by a combination of iron and cytokines can lead to a ferritin concentration of >20 000 µg/l in adult-onset Still's disease (*77,78*). In the reactive haemophagocytic syndrome there is an inappropriate activation of monocytes leading to haemophagocytosis and cytokine release. A ferritin concentration of up to 400 000 µg/l has been reported in children (*79,80*) and adults (*81*). Patients with acquired immunodeficiency syndrome (AIDS) may also have a reactive haemophagocytosis syndrome and high concentrations may also occur in AIDS sufferers with disseminated histoplasmosis (*82*).

FIGURE 1
A diagrammatic representation of a macrophage (left), plasma in the centre, and on the right, a liver parenchymal cell



Cytosolic ferritin (O) is released directly from damaged cell membranes into plasma or secreted (top) after synthesis on membrane bound polysomes and glycosylation (◎). In the circulation non-glycosylated ferritin may interact with ferritin binding proteins followed by removal of the complex from the circulation. Many cells also carry ferritin receptors, presumably for both secreted ferritin and cytosolic ferritin (see text). Injection of spleen ferritin into the circulation in man is followed by rapid uptake by the liver.
Adapted from Worwood (*83*), with permission of the publisher.

10.6 High serum ferritin concentrations and congenital cataract

An interesting cause of a high ferritin concentration in the absence of iron overload is associated with inherited cataract formation. In has now been demonstrated that mutations in the 'stem loop' structure of the ferritin L subunit may lead to synthesis of the 'L' subunit of ferritin that is no longer regulated by iron concentration (*84*). This causes an increase in the serum ferritin concentrations up to about 1 000 µg/l) in the absence of iron overload.

11. Biochemistry and physiology of plasma ferritin

11.1 Immunological properties and iron content

Plasma ferritin resembles liver or spleen ferritin immunologically and is recognised by polyclonal or monoclonal antibodies raised against these ferritins (see above). In patients with iron overload plasma ferritin has a relatively low iron content in purified preparations of 0.02–0.07 µg iron/µg protein (*85,86*) or a mean of 0.06 µg iron/µg protein when measured by immunoprecipitation (*87*). Purified horse serum ferritin has an iron content of <0.01 µg iron/µg protein (*88*). In the liver and spleen of patients with iron overload the iron content of ferritin is >0.2 µg iron/µg protein. Despite these findings several recent papers have indicated that serum ferritin has a much higher iron content. In 1997 ten Kate et al. (*89*) purified ferritin by immuno-precipitation and measured the iron content by atomic absorption spectrophotometry. They found a mean iron saturation of ferritin of 24% in normal serum giving a concentration of 0.13 µg iron/µg protein. They suggested that the extensive purification used in earlier studies had lead to a loss of iron, although this is unlikely unless reducing agents were present in the buffers used. Herbert et al. (*90*) claimed that the measurement of serum ferritin iron by a similar procedure provided an accurate assessment of the whole range of human body iron status, unconfounded by inflammation. Later Nielsen et al. (91) determined the iron content of serum ferritin derived from patients with iron overload and tissue damage using the method of ten Kate et al. (*89*). The iron saturation was about 5% and they found that the assay for ferritin iron was of little benefit in the diagnosis of iron overload. Yamanishi et al. (*92*) determined ferritin iron concentrations from 0.02 to 0.04 µg iron/µg ferritin protein in serum samples with ferritin concentrations greater than 2 000 µg/l. It should be pointed out that in 1956 Reissmann and Dietrich found that iron-rich ferritin was only detectable in the circulation after liver necrosis (*93*). Another consideration is that, in a normal subject with a serum iron concentration of 20 µmol/l and a serum ferritin concentration of 100 µg/l, the ferritin iron concentration would be only 1% of the transferrin iron concentration, even if the iron content is assumed to be high (0.15 g/µg protein). Clearly specific antibodies and the effective washing of the immunoprecipitate are essential if ferritin iron is to be detected.

11.2 Glycosylation

On isoelectric focusing both native and purified serum ferritin display a wide range of isoferritins covering the pI range found in human tissues (*85,94*) yet on anion exchange chromatography, serum ferritin is apparently a relatively basic isoferritin (*85*). The reason for this discrepancy and the heterogeneity of ferritin on isoelectric focusing appears to be glycosylation. In normal serum about 60% of ferritin binds to concanavalin A (*67*) whereas tissue ferritins do not bind. Incubation with neuraminidase converts the acidic ferritins of serum to the basic isoferritins but the pI of acidic heart ferritin is unaffected (*95*). A carbohydrate containing G subunit has also been identified in purified preparations of serum ferritin in addition to the H and L subunits (*86,96*).

11.3 Origin of serum ferritin and its clearance from the circulation

These findings suggest that some ferritin may enter the circulation by secretion, rather than by release from damaged cells. In hepatocytes there is direct evidence of

regulated secretion of glycosylated ferritin (*97*). Secreted ferritin may originate from phagocytic cells that degrade haemoglobin (Figure 1). When there is tissue damage, direct release of cytosolic ferritin through damaged cell membranes becomes important. In patients with ferritinaemia resulting from necrosis of the liver, the plasma ferritin shows reduced binding to concanavalin A (*67*). Findings in patients with haemophagocytosis or in the early stages of haemochromatosis confirm the importance of phagocytic cells (see below).

Another explanation for the differences between plasma and tissue ferritins may be differences in their clearance from the circulation. Plasma ferritin labelled with [^{131} I] was removed only slowly [T½<24h] from the plasma of normal subjects after intravenous injection (*98*) but spleen ferritin labelled in the same way was cleared very rapidly with a T½ of about 9 minutes (*99*). Such a rapid clearance may be due to interaction with ferritin receptors on hepatocytes (*100*) which appear to have a higher affinity for liver ferritin than for serum ferritin, at least in experiments on rats. Rapid clearance may also be initiated by interaction with ferritin binding proteins in the plasma (*101–104*). Several isoferritins may be released into the plasma but the ones which normally accumulate are L_{24} molecules and glycosylated molecules that are rich in L-subunits and again contain little iron. The L_{24} molecules take up iron slowly *in vitro* and have been termed 'natural apoferritin' (*105*). These molecules may accumulate in the plasma because their clearance by receptors, or their interaction with binding proteins, requires at least some H-subunits. The glycosylated protein may have little opportunity to acquire iron during secretion.

12. Red cell ferritin and its diagnostic use

The ferritin present in circulating erythrocytes is but a tiny residue of the amount in its nucleated precursors in the bone marrow. Normal erythroblasts contain ferritin which is immunologically more similar to heart ferritin than liver ferritin (i.e., ferritin rich in H-subunits) and mean concentrations are about 10 fg ferritin protein/cell (*106*). The concentration declines throughout the process of cell maturation and only about 10 ag/cell (10^{-18} g/cell) remains in the erythrocyte when measured with antibodies to L-ferritin, with a somewhat higher concentration detected using antibodies to H-type ferritin (*107,108*). Red cell ferritin concentration has generally been measured with antibodies to L-ferritin and reflects the iron supply to the erythroid marrow. The concentration tends to vary inversely with the red cell protoporphyrin concentration (*107*). Thus in patients with rheumatoid arthritis and anaemia, a low concentration is found in those with microcytosis, and a low serum iron concentration is observed regardless of the serum ferritin concentration (*109*). The red cell ferritin concentration does not therefore necessarily indicate the concentration of iron in storage. The red cell ferritin concentration may be useful to differentiate between hereditary haemochromatosis and alcoholic liver disease (*110*) and possibly to distinguish heterozygotes for haemochromatosis from normal subjects (*107*). The mean red cell ferritin content in patients with untreated inherited haemochromatosis was found to be about 70 times normal, and fell during phlebotomy. In some patients the concentration was still high after phlebotomy even when the serum ferritin concentration was within the normal range. This was shown to reflect the concentration of iron in liver parenchymal cells, which was still higher than normal (*110*). Furthermore the ratio of red cell ferritin (ag/cell) to serum ferritin (µg/l) was found to be about 0.5 in hereditary haemochromatosis but only 0.03 in patients with alcoholic

cirrhosis, thus clearly separating the two conditions. There may also be advantages of red cell ferritin over the assay of serum ferritin to estimate iron stores in patients with liver damage because the red cell ferritin concentration should not be greatly influenced by the release of ferritin from damaged liver cells. However, a high concentration of red cell ferritin is also found in individuals with thalassaemia (*111*,*112*), megaloblastic anaemia (*113*) or myelodysplastic syndromes (*108*) presumably indicating a disturbance of erythroid iron metabolism in these conditions.

Despite these specific diagnostic advantages (*114*) an assay for red cell ferritin has seen little routine application. This is because it is necessary to have fresh blood in order to separate the red from white cells, which have a much higher ferritin concentration.

13. Ferritin in urine

Although methods to estimate the concentration of ferritin in urine have been described and urine ferritin concentration is correlated with the concentration in serum (*115*,*116*) the technique has received little attention.

14. Assay of serum ferritin

Methods to prepare ferritin, raise antibodies to ferritin, and immunoassays for serum ferritin have been fully described (*33*) along with a reliable enzyme immunoassay (*117*,*118*). The serum ferritin assay is a routine measurement in most diagnostic laboratories and further discussion is not warranted. Several immunoassays for ferritin have been evaluated for the Medical Devices Agency of the UK (*119*,*120*). However some discussion of possible pitfalls and standardization is justified.

14.1 Samples

In many assays both plasma and serum give the same results but in some cases plasma collected in EDTA gives different values to serum. Samples may be stored at -20 °C or -80 °C for several years. Several rounds of freezing and thawing do not lead to changes in serum ferritin concentration, nevertheless freezing and thawing should be kept to a minimum.

14.2 Pitfalls

There are a number of theoretical and practical problems associated with the assay of serum ferritin. In theory, there may be problems because ferritin consists of a family of isoferritins which differ in subunit composition and thus in isoelectric point, and it is possible to generate specific antibodies which recognise particular isoferritins (see above). In practice, this has not been a problem because, in general, the ferritin found circulating in the plasma is similar to the L-rich ferritin found in liver or spleen (see above). A more practical concern is the very wide range in ferritin concentration that can be encountered in serum. In hospital patients the ferritin concentration can range from <1 µg/l in some patients with iron deficiency anaemia to in excess of 100 000 µg/l in patients with necrosis of the liver. The early two-site immunoradiometric assays suffered from a problem called the “high-dose hook” effect. In this situation a very high ferritin concentration could give readings in the lower part of the standard curve. In order to ensure that results were not artefactually low due to

the high dose hook effect, it is necessary to do the assay at two dilutions and to show that the greater dilution reduced the apparent ferritin concentration.

Interference by non-ferritin proteins in serum may occur with any method, but particularly with labelled antibody assays. Serum proteins may inhibit the binding of ferritin to the solid phase when compared with the degree of binding in buffer solution alone. Such an effect may be avoided by diluting the standards in a buffer containing a suitable serum, or by diluting serum samples as much as possible. For example, for two-site immunoradiometric assays, the sample may be diluted 20 times with buffer while the standards are prepared in 5% normal rabbit serum (if antibodies have been raised in rabbits) in buffer. Further dilution of the sera is then carried out with this solution. Some methods give low recoveries of ferritin from plasma collected in EDTA, and the use of the plasma samples should therefore be investigated carefully.

Another problem has occurred because antibodies to some animal proteins are sometimes present in human serum. These can interfere with the assay of serum ferritin, giving a spuriously high ferritin concentration (*121*).

14.3 Standardization

The serum ferritin assay is often used in epidemiological surveys in which the iron status of populations is examined. For such an assay to be comparable between surveys it is important that a common standard is used and that the assays are calibrated against this. The first WHO standard for the assay of serum ferritin was introduced in 1990 (reagent 80/602), and was replaced by the second international standard in 1993 (reagent 80/578). A third international standard, which is a recombinant ferritin, is now in use (reagent 94/572). The introduction of an international standard has led to considerable improvements in the standardization of the assay for ferritin (*11,122*) but differences in reference ranges were still being reported (*123*) and there are problems with comparisons during longitudinal studies (see later). Almost all commercial assays in current use have been calibrated against either the first or second international standard, although these have not been available for some years.

The United Kingdom National External Quality Assessment Scheme (UK NEQAS) has recently investigated the recovery of the third international standard for assay systems used by UK participating laboratories. In most cases recoveries were acceptable and ranged from 90 to 110%. However in two cases recoveries were 69% and 157%. Despite this, the performance of the assays of ferritin in serum samples in the NEQAS haematinics scheme was considered to be acceptable (*124*). The authors concluded that these results call into question the suitability of the third international standard for calibration. However, another concern is that none of the assays appeared to have been calibrated against the current standard which has been used for the last ten years.

Discrepancies remain in the definition of normal ranges of serum ferritin concentration. Some manufacturers define a normal range as the ferritin concentration found in unselected, apparently normal subjects. However, a proportion of the normal population have almost no storage iron without being anaemic, particularly young women, and a smaller proportion will be anaemic. The “normal range” in young females will thus include ferritin concentrations found in iron deficiency. This confusion between “normality” and iron deficiency causes difficulties in the interpretation of ferritin concentrations.

Evaluation of several commercial assay systems suggests that most are free from problems of protein interference or high dose "hook effects" and are well standardized (*119*), but these points should be investigated before adopting a system for routine use.

15. Methodological and biological variability of measures of iron status

Assays of blood for indicators of iron status vary greatly in both methodological and biological stability. Haemoglobin concentrations are stable and a simple and well-standardized method ensures a relatively low day to day variation in individuals (Table 3). Automated cell counters can analyse at least 10 000 cells and thus reduce errors. The more complicated procedures involved in immunoassays lead to a greater variation in ferritin assays, with a coefficient of variation of at least 5%. This variation, coupled with some physiological variation, gives an overall coefficient of variation for serum ferritin for an individual over a period of weeks of the order of 15%. There is however little evidence of any significant diurnal variation in serum ferritin concentration (*125*). There is no information on seasonal factors influencing most of these analyses, although seasonal change in red cell parameters have been reported (*126*).

The effect of menstruation on indicators of iron status was examined in 1712 women aged 18-44 years during the Second National Health and Nutrition Examination Survey (NHANES II) after adjusting for potential confounders. Adjusted mean values of haemoglobin (Hb), transferrin saturation (TS), and serum ferritin (SF) concentration were lowest for women whose blood was drawn during their menses and highest for women examined in the luteal or late luteal phase of their menstrual cycle (Hb = 130 vs 133 g/l; TS = 21.2% vs 24.8%, $P < 0.01$ for both; and SF = 17.2 vs 24.0 µg/l, $P < 0.05$). The prevalence estimate of impaired iron status was significantly higher for women whose blood was drawn during the menstrual phase than for women whose blood was drawn during the luteal and late luteal phases. The authors concluded that cyclical variations in indicators of iron status are a potential source of error when iron status is assessed in surveys of large populations that include women of reproductive age (*127*).

Results from a number of studies of overall variation are shown in Table 3, but it should be noted that the type of blood sample, the length of study period, and the statistical analysis performed, vary from study to study. The somewhat higher variability in the haemoglobin and ferritin concentration reported by Borel et al. (*128*) may be due to their use of capillary blood and plasma. Pootrakul et al. (*129*) have demonstrated that the mean plasma ferritin concentration is slightly higher in capillary blood specimens than in venous specimens and that the variation within and between samples was approximately three times greater. Variability was less in capillary serum but still greater than venous serum. However the increased variability of capillary samples may be related to the blood sampling technique as Cooper and Zlotkin (*130*) found little difference in variability between venous and capillary samples.

Starvation or even fasting for a short period can cause an increase in the serum ferritin concentration (*131*) while a vitamin C deficiency may reduce the ferritin concentration (*70*). Moderate exercise has little effect on serum ferritin concentration

(*132*) although exhaustive exercise leads to an increase in serum ferritin concentration due to muscle damage and inflammatory reactions (*133,134*).

These variations have clear implications for the use of these assays in population surveys (*3,135*) or in the assessment of patients (*128*). For accurate diagnosis either a multi-parameter analysis is required or the assay of several samples (see below).

TABLE 3
The coefficient of variation (%) reported in assays of iron status (within-subject, day-to-day variation in otherwise healthy subjects)

Haemoglobin	Serum ferritin	Serum iron	Total iron binding capacity	Zinc protoporphyrin	Serum transferrin receptor	Reference
–	15 (MF)	–	–			Dawkins et al. (*125*)
2 (F)	15 (MF)	–	–			Gallagher et al. (*136*)
–	–	29(F)	–			Statland and Winkel (*137*)
–	–	27 (M)	–			Statland et al. (*138*)
3 (MF)	–	–	–			Statland et al. (*139*)
–	15 (MF)	29 (MF)	–			Pilon et al. (*140*)
–	13 (MF)[a]	33 (MF)[a]	11 (MF)[a]			Romslo and Talstad (*141*)
4 (MF)	14 (M) 26 (F)	27 (M) 28 (F)	– –			Borel et al. (*128*)
–	27 (MF)	29 (MF)	7 (MF)		14 (MF)	Maes et al. (*142*)
– –	26 (F)[b] 15 (M)[b] 28 (F)[c] 12 (M)[c]				14 (F) 12 (M) 11(F) 10 (M)	Cooper and Zlotkin (*130*)
–	–	–	–	5 (MF)	–	Hastka et al. (*143*)
3 (F)[d]	11 (F)[d]	26 (F)[d]	4 (F)[d]		13 (F)[d]	Ahluwalia (*144*)

M, males only; F, females only; MF, males and females
[a] Anaemic patients
[b] Venous blood
[c] Capillary blood
[d] 70–79 years old healthy women

16. The predictive value of indicators of iron metabolism

Despite years of investigation there is little reliable comparative information on indicators that will distinguish reliably between the presence and absence of storage iron. Most investigators have used the grade of stainable iron in the bone marrow as a "gold standard". This involves an invasive procedure and so limits greatly the number of patients that can be investigated. It is often difficult to justify bone marrow aspiration to determine the iron status of a patient, and it is even more difficult in the case of normal volunteers. Furthermore, the examination of a stained bone marrow aspirate is not a reproducible procedure as there can be observer error (*145*) while inadequate specimens and a lack of correlation with response to iron therapy have been described (*146*). An alternative is to demonstrate a change in haemoglobin concentration in response to oral iron therapy, and this has been the method of choice in paediatric practice.

16.1 Iron deficiency anaemia in adults

Iron deficiency anaemia (IDA) in adults occurs typically due to a gradual decline in the iron content of the body due to a loss of haemoglobin and a depletion of iron stores. In the absence of malabsorption there is a good response to iron therapy. Menstrual blood loss is the most common cause of IDA in pre-menopausal women, but blood loss from the gastro-intestinal tract or malabsorption of iron are common causes in men and post-menopausal women. People with asymptomatic colonic or gastric carcinoma may present with IDA and it is essential to exclude these conditions as the cause of anaemia in elderly men and women (*147*).

Early studies of patients with iron deficiency anaemia characterised by microcytic anaemia, low serum iron concentration, high total iron binding capacity (TIBC), and either an absence of stainable iron in the bone marrow or who had a subsequent response to therapeutic iron, showed that serum ferritin concentrations were less than 12–16 µg/l (1). Hallberg et al. (*148*) determined the serum ferritin concentration of 203 women aged 38 years who had undergone bone marrow examination. They concluded that a value of <15 µg/l was the best predictor of iron deficiency (confirmed by an absence of stainable iron in the bone marrow) and noted that this threshold was similar to one derived from earlier population surveys and studies of clinical cases (*148*).

Almost all measures currently used to assess iron status show a high sensitivity and specificity in distinguishing between subjects with iron deficiency and those with iron stores and a normal haemoglobin concentration, but only in the absence of any other disease process. Zanella et al. (*149*) examined the sensitivity and predictive value of serum ferritin and zinc protoporphyrin (ZPP) concentrations to identify iron deficiency. Iron deficiency anaemia was defined as a haemoglobin concentration below 13.5 g/dl for men and 11.8 g/dl for women, a transferrin saturation below 16%, and an increase in haemoglobin concentration after oral iron therapy (*149*). The subjects with iron deficiency but without anaemia were blood donors whose haematological profile over time indicated possible iron deficiency and whose haemoglobin concentration improved in response to iron supplementation. The overall sensitivity and specificity of diagnosis were 82% and 95% for serum ferritin and 61% and 95% for ZPP. However while the sensitivity was over 90% for both ferritin and ZPP in cases of severe anaemia, in the absence of anaemia the sensitivity dropped to 70% for ferritin and less than 50% for ZPP. In a systematic review of the diagnostic value of various laboratory tests to diagnose iron deficiency it was concluded that serum ferritin was the most powerful test for simple iron deficiency in both populations and hospital patients. However this analysis did not include the transferrin receptor (*150*).

16.2 Detection of iron deficiency in acute or chronic disease

In practice there are two different questions: first, identifying patients with inflammation, infection, malignancy and renal failure and an absence of storage iron in reticuloendothelial cells; and second, identifying a functional iron deficiency in which there is an inadequate iron supply to the bone marrow in the presence of storage iron in reticuloendothelial cells. This problem is usually encountered among patients with renal failure receiving erythropoietin to correct anaemia.

Table 4 summarises a number of studies in which bone marrow iron has been determined and the sensitivity and specificity of various assays to determine iron status have been compared. Despite varying results between studies, some general points may be made.

TABLE 4

The sensitivity/specificity of methods to diagnose iron deficiency in the presence of chronic disease[a]

	Reference											
	Van Tellingen et al. (*156*)	Lee et al. (*157*)[b]	Mast et al. (*158*)	Kotru et al. (*152*)	Punnonen et al.(*159*)[b]	Baumann Kurer et al. (*160*)	Bultink et al. (*161*)	Means et al. (*162*)	Joosten et al. (*163*)	Balaban et al. (*164*)	Mast et al. (*165*)	Fernandez-Rodriguez et al. (*166*)
Test												
Mean cell volume	–	–	0.32/0.93	L	0.86	–	–	0.42/0.83	–	–	–	–
Mean cell Haemoglobin	–	–	–	–	–	0.71/0.71	–	–	–	–	–	–
% hypo	–	–	–	–	–	0.77/0.90	–	–	–	–	–	–
Reticulocyte haemoglobin concentration	–	–	0.73/0.74	–	–	–	–	–	–	–	–	–
Serum iron concentration	–	–	–	L	0.68	L	–	–	NS	–	–	–
Total iron binding capacity	–	–	–	L	0.84[c]	L	–	–	–	–	–	< 0.65[b]
Percentage saturation	–	–	0.65/0.70	L	0.79[d]	L	–	0.38–0.89	–	–	–	< 0.65[b,c]
Serum ferritin concentration	0.79/0.97	0.870	0.52/0.93	90/75	0.89	0.86/0.90	1.00/0.81	0.25/0.99	0.94/0.95	0.60/0.90	0.92/0.98	0.83b (0.75/0.75)
Red cell ferritin concentration	–	–	–	L	–	–	–	–	–	0.82/0.83	–	0.68[b]
Zinc protoporphyrin	0.74/0.94		–	–	–	L	–	–	–	–	–	–
Serum transferrin receptor concentration	0.63/0.81	0.704	–	–	0.98	L	1.00/0.84	0.71/0.74	0.61/0.68	–	0.92/0.84	0.69[b]
Serum transferrin receptor concentration to log ferritin concentration	0.74/0.97	0.865	–	–	1.00	–	1.00/0.97	0.67/0.93	–	–	–	–

L, Lower sensitivity/specificity than serum ferritin, individually or in combination. The combination of ferritin and erythrocyte sedimentation rate or C-reactive protein did not improve efficiency.

[a] Adults, iron stores determined by staining for iron in bone marrow). Optimum diagnostic thresholds selected vary.

[b] Area under Receiver operating characteristics (ROC) curve

[c] Transferrin concentration (equivalent to TIBC, see text)

[d] Transferrin Index (equivalent to % saturation, see text)

Conventional red cell parameters such as the haemoglobin concentration, mean cell volume (MCV), mean cell haemoglobin (MCH), and reticulocyte counts do not distinguish between the presence or absence of bone marrow iron in patients with chronic disease.The serum iron concentration is almost invariably low in patients with chronic disease and although the TIBC (or transferrin concentration) is higher in patients with no storage iron, neither this measurement nor the transferrin saturation derived from the serum iron and TIBC, provide useful means of identifying patients with iron deficiency. In patients with chronic disease the serum ferritin concentration reflects the concentration of storage iron, but the concentration of ferritin is higher than in healthy subjects. For this reason it is necessary to set a threshold of 30–50 µg/l of ferritin in order to distinguish between the presence and absence of storage iron. Even when this threshold is applied the sensitivity of diagnosis is low.

Combinations of serum ferritin, ESR or CRP either in a discriminant analysis (*151*) or logistic regression (*152*) provide only marginal improvement in the ability to detect a lack of storage iron.

The serum transferrin receptor concentration is usually raised in patients with chronic disease and an absence of storage iron in the bone marrow although there is disagreement as to whether or not the assay is superior in predictive power than the serum ferritin concentration. Several studies show that the ratio of sTfR/$\log_{10}$ SF is superior to either test on its own. The use of the log of serum ferritin in this ratio decreases the influence of the acute phase response on the ferritin component of the ratio. Although the $\log_{10}$ (sTfR/SF) ratio is an excellent measure of iron stores in healthy subjects (*153*) its use may not be not appropriate for clinical diagnosis of iron deficiency. When the assay of sTfR is easily done on an immunoanalyser the sTfR/log SF ratio may provide the best means of identifying the co-occurrence of iron deficiency in chronic disease. However if this ratio is to gain wide acceptance, this will also require the standardization of units and ranges for the various sTfR assays.

Measurements of the percentage of hypochromic erythrocytes or the reticulocyte haemoglobin concentration also provide some power to identify people with iron deficiency, but their sensitivity and specificity are not sufficiently high enough to distinguish between the presence and absence of storage iron in acute or chronic disease.

Patients with a functional iron deficiency will require parenteral iron therapy in order to respond to erythropoietin with an acceptable rise in haemoglobin concentration. The percentage of hypochromic erythrocytes has been shown to be a good predictor of a response (*154*) and Table 5). Fishbane et al. (*155*) concluded that the reticulocyte haemoglobin concentration (CHr) was a markedly more stable analyte than serum ferritin or transferrin saturation, and that it predicted functional iron deficiency more efficiently. Fishbane et al. (*155*) did not include the percentage of hypochromic cells in their analysis.

16.3 Iron deficiency in infancy and childhood

Diagnostic thresholds for iron deficiency and iron deficiency anaemia in infants are not universally agreed upon. There are rapid changes in iron status in the first year of life as the fetal haemoglobin is replaced by haemoglobin A. The serum ferritin concentration is a less useful guide to iron deficiency than in adults partly because of the changes in concentration that continue for the first 18 months (*167*) and the low concentrations generally found in children. In a study of healthy, breastfed infants

TABLE 5
The threshold of assays used to predict a response to treatment with intravenous iron in haemodialysis patients treated with erythropoietin, and the efficiency of the threshold in predicting a response[a]

Assay	Threshold	Efficiency (%)[b]
Hypochromic erythrocytes	>6%	89.6
Reticulocyte haemoglobin concentration	≤29 pg	78.4
Soluble transferrin receptor concentration	>1.5 mg/l	72.4
Erythrocyte zinc protoporphyrin concentration	>52 µmol/mol haem	73.0
Transferrin saturation	19%	70.4
Serum ferritin concentration	<50 µg/l	64.0

[a] Not all cell counters detect hypochromic red cells but this study indicates that the other tests have insufficient predictive power to justify cost beyond the blood count.
[b] Number of true positive and negative results/total number of tests
Adapted from Tessitore et al. (*146*), with permission of the publisher.

in Honduras and Sweden, Domellof et al. (*168*) evaluated the change in haemoglobin concentration in response to oral iron supplementation. At 4–6 months of age the initial haemoglobin concentration did not predict a response to iron therapy. At 6 months of age the haemoglobin concentration, the MCV and the ZPP concentration predicted the response, but SF and sTfR did not. Table 9 shows the proposed thresholds of ferritin concentration to diagnose iron deficiency in infants. In some studies the ferritin concentration has been assayed in addition to MCV, zinc protoporphyrin, transferrin saturation or serum transferrin receptor, in order to apply a multiple parameter approach to diagnosing iron deficiency and iron deficiency anaemia (see for example (*169*,*170*).

Margolis et al. (*171*) found that, in children age 6 months to 17 years, the best predictor of a response was the initial haemoglobin concentration, although the sensitivity of this parameter was only 66% and the specificity 60%. The concentration of SF, TS and erythrocyte protoporphyrin had even lower efficiencies, while a combination of the various measures made little improvement. Hershko et al. (*172*) studied children aged 1–6 years from villages in the Golan Heights and concluded that erythrocyte protoporphyrin was a more reliable index of iron deficiency than serum ferritin and serum iron. They suggested that a significant incidence of chronic disease affected both ferritin and iron values. The concentration of ZPP provides a useful indicator of iron-deficient erythropoiesis although high values may indicate lead poisoning rather than iron deficiency. The small volume of blood needed to measure ZPP is also an advantage in paediatric practice.

A recent report confirms the effect of low level infection on measures of iron status. Abraham et al. (*64*) studied 101 healthy, 11 month old infants. On the morning of blood sampling, slight clinical signs of an respiratory tract infection were observed in 42 infants. Extensive blood analyses were done, including a highly sensitive assay for CRP. While the concentration of CRP measured using routine methods gave values of <6 mg/l for all infants, the highly sensitive assay gave values that were higher for many of the infants with symptoms of infection. The serum iron concentration was low in these children and was significantly correlated with the CRP concentration. In those children for whom a second blood sample was taken, the serum ferritin concentration was higher in the sample with the higher CRP concentration and the serum iron concentration was reduced, but the sTfR and transferrin concentrations were unchanged.

16.4 Treatment of iron deficiency anaemia

Oral iron therapy given at conventional doses of 60 mg of iron, 3 times daily, has little immediate effect on the serum ferritin concentration, which rises slowly as the haemoglobin concentration increases. However with double doses there is a rapid rise in serum ferritin concentration in a few days to within the normal range, an increase that probably does not represent an increase in storage iron (*173*). Intravenous iron causes a rapid rise in serum ferritin concentration to a concentration that may be above the normal range, but it gradually drops back to normal (*174*).

16.5 Screening blood donors for iron deficiency

A number of studies have shown that regular blood donation reduces storage iron levels (see reviews by Skikne et al. (*175*) and Milman and Kirchhoff (*37*)). The conventional screening test for anaemia, the "copper sulphate" test, lacks specificity so that donors may be deferred unnecessarily. Despite the availability of the serum ferritin assay for 30 years there has been little attention to the fundamental relationship between storage iron levels and the ability to donate blood. Screening blood donors by routinely assaying serum ferritin may make it possible to predict the development of iron deficiency anaemia (37) and may identify donors with high iron stores who may give blood more frequently than is usually permitted. However the assay has low predictive power to identify donors who are homozygous for HFE gene C282Y (see below).

16.6 Pregnancy

In early pregnancy the serum ferritin concentration usually provides a reliable indication of the presence of iron deficiency. Haemodilution in the second and third trimesters of pregnancy reduces the concentration of all measures of iron status and means that the threshold values for iron deficiency established in non-pregnant women are not appropriate. In principle the calculation of values as ratios, such as ZPP µmol/mol haem, transferrin saturation or sTfR/ferritin, should be more diagnostically reliable. In a study of healthy, non-anaemic women supplemented with iron (176), the serum iron, TS and SF concentrations fell from the first to the third trimester and increased after delivery while TIBC increased during pregnancy and fell after delivery. The sTfR concentration showed a substantial increase of about 2-fold during pregnancy, a change that probably reflects increased erythropoiesis (*176*). In contrast, Carriaga et al. (*177*) reported that the mean sTfR concentration of pregnant women in their third trimester did not differ from that in non-pregnant women, and that the sTfR concentration was not influenced by pregnancy per se. Choi et al. (*176*) suggest that the use of different assays and the different ages of subjects in the control groups may explain this discrepancy. In practice the Institute of Medicine (USA) has recommended that iron deficiency in the first and second trimester can be identified by a haemoglobin concentration <110 g/l and a ferritin concentration <20 µg/l (*178*). Women with low iron stores only should receive lower doses of oral iron than those who are also iron deficient and anaemic. In the third trimester all women should receive iron supplements. Other US guidelines recommend supplementation for all women throughout pregnancy (*179,180*).

16.7 Genetic haemochromatosis

Iron overload in haemochromatosis begins with enhanced iron absorption leading to an increase in plasma iron concentration and transferrin saturation followed by an increasing iron concentration in liver parenchymal cells. As the serum ferritin concentration reflects the iron concentration of macrophages (*10*), and macrophages accumulate little iron initially, the serum ferritin concentration might be expected to remain normal during the early stages of iron accumulation. The transferrin saturation is therefore the most efficient test for detecting iron accumulation in genetic haemochromatosis (*181*). The use of serum ferritin to detect genetic haemochromatosis in populations is discussed later.

16.8 Secondary iron overload

The major aims of diagnostic tests are to estimate the degree of iron overload and to monitor the success of chelation therapy in removing iron from the body. The only reliable methods are the quantification of the amount of iron in the liver using biopsy samples, an invasive procedure, or by measuring magnetic susceptibility (*182*) which can only be done in three countries. In practice, the serum ferritin concentration provides a combined index of storage iron concentration and liver damage, and gives useful information to monitor the progress of treatment. The aim of treatment with chelating agents is to reduce both the tissue iron concentration and tissue damage. The reduction of the concentration of ferritin in serum below 1 000 µg/l is a realistic aim. This topic has recently been reviewed (*183*).

17. Population studies

17.1 Distribution of serum ferritin concentration in people in the United States of America and Europe

The first studies of serum ferritin concentrations in normal subjects including neonates, infants, children and adults, were drawn from populations in the USA, Canada, UK and Denmark (*1*). The sample sizes were usually relatively small and criteria to exclude people with iron deficiency or chronic disease etc. were not usually applied. The distribution of values has been summarized in several ways including the mean and standard deviation, although the typically right-skewed distributions of serum ferritin means that such parameters are inappropriate. Other ways of presenting the distribution include the median and range, median, 5th and 95th percentiles and geometric means. Much larger sample sizes were made available from the National Health and Nutrition Examination Surveys (NHANES) II and III. These surveys were designed to assess the health and nutritional status of the civilian, non-institutionalised population of the USA.

17.1.1 Age, sex and race

Figure 2 shows the distribution of the serum ferritin concentration and transferrin saturation of 20 040 men and women aged 17 to over 90 years using data from NHANES III (*184*). The analysis was limited to white (n = 8477), black (n = 5484) and Hispanic (n = 5304) subjects as only 775 people were of 'other' races. The values shown are the geometric means for each age group. The minimum number of unweighted observations in each 10 year age category (except for 17–19 years) was 30

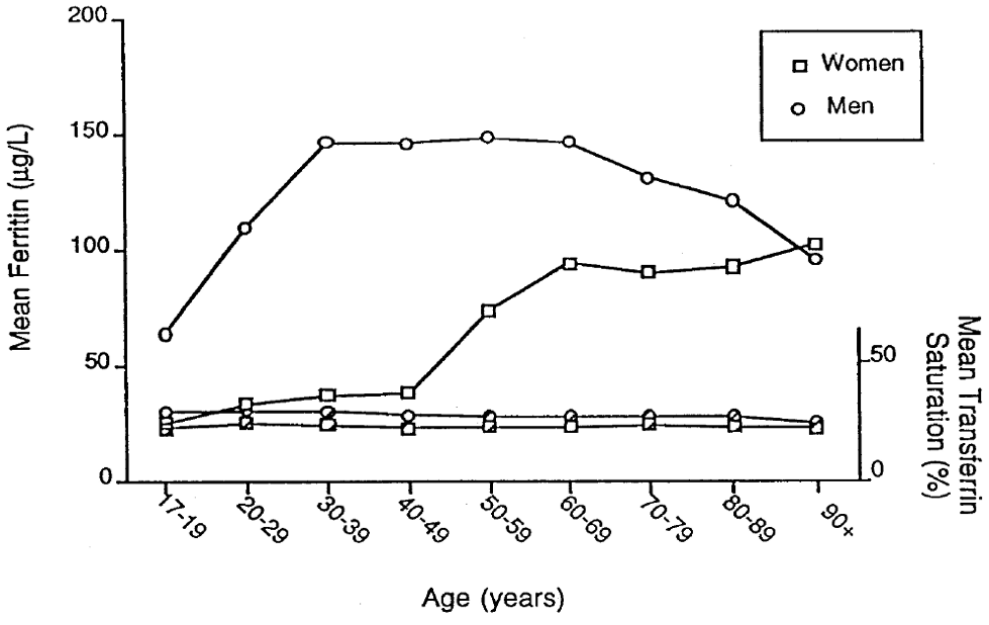
and analyses were weighted to adjust for the probability of selection due to sample design and non-response.

Figure 3 shows the distribution of the serum ferritin concentration by age and race. The geometric mean ferritin concentration in men was always higher in blacks than in whites or Hispanics. In women, the values for blacks were higher than the other groups after the menopause. It is unlikely that the higher mean values of serum ferritin concentration in US blacks are due to a greater intake of dietary iron. Popkin et al. (*185*) and Zacharski et al. (*184*) suggested that these high ferritin concentrations may indicate that higher iron stores contribute to the higher mortality and morbidity rates among blacks when compared with whites for many common diseases. However, it may be that higher morbidity is the cause of the higher ferritin concentrations later in life.

An alternative approach to examining the distribution of the serum ferritin concentration in a population was described by Custer at al. (*47*). Data were collected on over 964 000 patients unselected by race and medical condition. Over 98% of samples came from physicians' offices or blood-drawing stations. Information included results on 29 tests as well as age and sex. They analysed the distribution of serum ferritin according to age and sex for all subjects and then for a subgroup of 22 464 males and 37 450 females who had normal values for the other 28 assays. The concentration of ferritin was lower in the normal subgroup than in all subjects combined. Table 6 shows the 2.5th, 50th and 97.5th percentiles of the ferritin concentration for the subgroup of normal males and females.

FIGURE 2

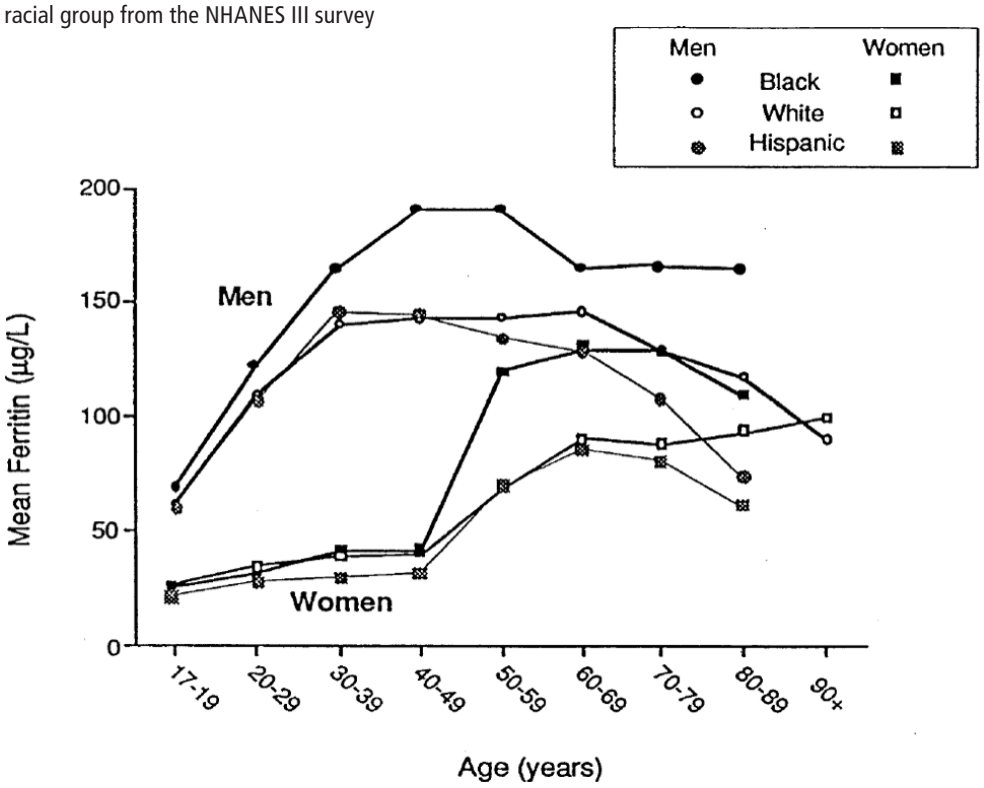
The distribution of mean serum ferritin concentration (left y-axis) and transferrin saturation (right y-axis) by age group for American men and women participating in NHANES III surveys



Adapted from Zacharski et al. (*184*), with permission of the publisher.

FIGURE 3

Distribution of mean serum ferritin concentration by age group for American men and women by age and racial group from the NHANES III survey



Adapted from Zacharski et al. (*184*), with permission of the publisher.

TABLE 6

The values of serum ferritin concentration (µg/l) by percentiles in males and females with normal laboratory test results for indicators of iron deficiency (other than ferritin)

Age groups (years)	Males				Females			
	Number	2.5%	50%	97.5%	Number	2.5%	50%	97.5%
12–<16	129	11	36	117	563	7	24	110
16–<20	616	17	56	171	1 143	6	27	100
20–<24	720	20	90	259	1 711	8	32	128
24–<28	1 085	25	105	311	2 175	7	35	140
28–<32	1 670	26	117	343	2 599	7	39	174
32–<36	1 508	23	123	385	3 007	7	38	174
36–<40	1 581	24	127	399	3 085	6	38	190
40–<44	1 734	19	122	427	3 241	7	38	193
44–<48	1 734	19	127	450	3 013	7	37	216
48–<52	1 657	19	128	481	2 457	7	41	232
52–<56	1 550	19	121	477	2 101	10	49	264
56–<60	1 463	20	127	486	1 826	13	60	308
60–<64	1 432	17	122	441	1 896	13	75	346
64–<68	1 541	14	120	488	1 873	13	80	369
68–<72	1 451	14	113	522	1 947	12	81	379
72–<76	1 148	15	111	485	1 718	12	78	381
76–<80	813	14	98	441	1 310	11	79	382
80–<90	766	16	107	474	1 523	13	76	369
Total	22 338				37 188			

Adapted from Custer et al. (*47*), with permission of the publisher.

17.1.2 Genes modifying iron status

Mutations of the HFE gene are common in Northern European populations (10–20% carrying C282Y and 20–30% H63D). From 0.25–1 % of the population will be homozygous for HFE C282Y and are at risk from iron overload (*186*). About 2 % will be compound heterozygotes (C282Y/H63D) and are at lesser risk. However, although most subjects who are homozygous for C282Y will accumulate excess iron the clinical penetrance is low and for compound heterozygotes is even lower. In population surveys, slightly but significantly higher values for serum iron and transferrin saturation have been found in heterozygotes for either C282Y (*187–190*) or H63D (*187,189,190*) with subjects lacking these mutations. The differences in ferritin levels were smaller and not significant except that Jackson et al. (*190*) found higher levels of serum ferritin in men heterozygous for C282Y. In compound heterozygotes and subjects homozygous for H63D there are greater differences (*189–191*). In heterozygotes for C282Y (*188,189*) and H63D (*189*) Hb levels were slightly higher than in subjects lacking mutations. Beutler et al. (*189*) noted a lower prevalence of anaemia among women carrying either mutation, but the differences were small and only significant if all subjects carrying mutations were compared with those lacking mutations. Serum ferritin concentrations in haemochromatosis may not accurately reflect tissue iron concentrations during the early stages of iron accumulation, particularly in heterozygotes. Serum ferritin concentrations are related to the levels of ferritin iron in macrophages and in haemochromatosis the iron initially accumulates in hepatic parenchymal cells. Edwards et al. (*192*) noted that liver iron concentrations in heterozygote family members were above the reference range although serum ferritin concentrations were not elevated. Thus iron accumulation may be underestimated in heterozygotes. Tables 7 and 8 illustrate population data for blood donors from South Wales.

Rare causes of genetic haemochromatosis include mutations in the HAMP (hepcidin) gene (*193,194*), transferrin receptor 2 (*195*), ferroportin 1 (*196–198*) and HFE2 (*199*) genes. Other genes may modify the phenotype involved in haemochromatosis. It was found by Langlois et al. (*200*) that men with the 2-2 haplotype for haptoglobin had a higher serum ferritin concentration than those with HP 1-1 or 2-1 but others have not confirmed this finding (*201–203*). Until now, no mutations in genes coding for iron transport or storage have been linked to iron deficiency.

TABLE 7
The mean (SD) of indicators of iron status for different genotypes among male blood donors

Genotype[a] Number of donors (%)	Serum iron concentration (µmol/l)	Unsaturated iron binding capacity (µmol/l)	Total iron binding capacity (µmol/l)	Transferrin saturation (%)	Serum ferritin concentration (µg/l)[b]
All donors 4952 (100%)	16.7 (6.4)	39.2 (11.2)	55.8 (10.6)	30.0 (12.1)	81 (24–196)
HHCC 2896 (58.5%)	16.0 (6.1)	40.8 (11.0)	56.8 (10.6)	28.3 (11.0)	79 (23–193)
HDCC 1167 (23.6%)	17.4 (6.5)***	38.2 (10.5)***	55.6 (10.4)**	31.8 (11.7)***	81 (23–197)
HHCY 644 (13.0%)	17.3 (6.0)***	35.8 (10.0)***	53.1 (10.0)*	33.1 (11.5)***	82 (29–201)*
DDCC 105 (2.1%)	19.1 (6.9)***	34.4 (10.5)***	53.5 (10.1)**	36.4 (13.1)***	99 (33–210)***
HDCY 111 (2.2%)	20.2 (6.9)***	31.2 (12.1)***	51.4 (11.6)***	40.5 (15.5)***	107 (24–260) ***
HHYY 29 (0.59%)	27.5 (9.9)***	15.6 (8.4)***	43.1 (8.3)***	63.6 (17.8)***	154 (50–410)***

Significance of difference from wild-type donors * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ (Mann-Whitney test for significance)
[a] HHCC, wild-type; HHYY, homozygous C282Y; DDCC etc, homozygous H63D
[b] Median (95% range).
Adapted from Jackson et al. (*190*), with permission of the publisher.

TABLE 8
The mean (SD) of indicators of iron status for different genotypes among female blood donors

Genotype[a] Number of donors (%)	Serum iron concentration (µmol/l)	Unsaturated iron binding capacity (µmol/l)	Total iron binding capacity (µmol/l)	Transferrin saturation (%)	Serum ferritin concentration (µg/l)[b]
All donors 5372 (100%)	14.3 (6.3)	42.9 (12.9)	56.8 (11.2)	25.4 (12.4)	44 (9–130)
HHCC 3100 (57.7%)	13.8 (6.1)	44.2 (12.1)	58.1 (11.4)	24.6 (10.9)	44 (9–126)
HDCC 1277 (23.8%)	14.7 (6.2)**	41.5 (12.2)***	56.2 (10.9)***	27.0 (12.0)***	43 (10–127)
HHCY 682 (12.7%)	14.6 (6.1)*	40.7 (12.5)**	55.1 (11.5)***	27.3 (12.1)***	44 (8–116)
DDCC 135 (2.5%)	15.6 (7.1)**	38.4 (11.8)***	54.0 (11.1)***	29.5 (13.8)***	46.5 (11–140)
HDCY 138 (2.6%)	16.6 (6.8)***	34.2 (10.5)***	50.8 (8.6)***	33.2 (14.0)***	50 (10–180)*
HHYY 41 (0.76%)	21.4 (8.4)**	23.4 (12.6)***	44.3 (10.4)***	49.9 (20.5)***	65 (8–238)**

Significance of difference from wild-type donors * $p < 0.01$, ** $p < 0.001$,*** $p < 0.0001$ (Mann-Whitney test for significance)
[a] HHCC, wild-type; HHYY, homozygous C282Y; DDCC etc, homozygous H63D
[b] Median (95% range)
Adapted from Jackson et al. (*190*), by permission of the publisher.

17.2 Prevalence of iron deficiency

Iron deficiency has generally been defined as an absence of iron stores while iron deficiency anaemia as the same but with a haemoglobin concentration below a defined threshold. The absence of stored iron may simply be defined as a serum ferritin concentration <15 µg/l (see below). However an analysis of NHANES II data revealed that for those subjects with either a low ferritin concentration or transferrin saturation, the prevalence of anaemia was only slightly greater than in those with normal test results. Iron deficiency was therefore defined as two abnormal results out of a panel of three: free erythrocyte protoporphyrin concentration, serum ferritin concentration, and transferrin saturation (*42*). Not surprisingly the results produced by the two diagnostic approaches were very different. Hallberg et al. (*148*) estimated the prevalence of iron deficiency among women using a serum ferritin concentration of <15 µg/l to be about 30% in the USA (NHANES II) and 33% in Sweden. Using the results of "two of three tests abnormal" approach on data from NHANES II and III, about 10% of young women were iron deficient but only 2–5% had iron deficiency anaemia (*3*).

These different diagnostic approaches may not entirely explain the very different prevalences of iron deficiency reported in Sweden and the USA. Among pre-menopausal women surveyed in Denmark in both 1984 and 1994 approximately 11% were iron deficient (defined as a serum ferritin concentration <16 µg/l) while 2.7% had iron deficiency anaemia – prevalences very much closer to those in the USA than for Sweden. Throughout Europe between 11 and 45 % of menstruating women were reported to have a serum ferritin concentration below thresholds varying from 10 to 17 µg/l (*204*).

17.3 Longitudinal and intervention studies

For the last 30 years, serum ferritin has been an important tool for investigating changes in storage iron concentration. However attempts to measure the changes in iron stores over several years or a decade have been bedevilled by changes in assay methods, by changes in survey procedures (*205*), or changes in other confounding factors such as the blood lead concentration (*206,207*). Milman et al. (*207,208*) have compared changes in iron stores and the prevalence of iron deficiency and iron overload in a cohort of Danish men and women studied in 1984 and 1994. In 1987 the fortification of flour with iron was abolished, thereby reducing daily iron intake significantly. Surprisingly perhaps, the prevalence of iron deficiency or iron deficiency anaemia (ferritin <13 µg/l and a haemoglobin concentration <5th percentile) did not increase, but in elderly women and men the prevalence of iron overload (ferritin >300 µg/l) increased. Changes in the ferritin assay procedure required the correction of values to permit valid comparisons to be made. Changes in diet, alcohol consumption and smoking habits were probably responsible for these findings.

17.4 Iron stores in people in developing countries

In many regions where iron deficiency is of great public health importance malaria, hookworm and other parasitic infections are endemic. Whether or not serum ferritin is a valid indicator of iron stores depends on the type and degree of infection. Where malaria is "holo-endemic" serum ferritin appears to be little affected by parasite load (209). However in malarial disease a high ferritin concentration results from the destruction of red blood cells, an acute phase response, suppressed erythropoiesis, and ferritin released from damaged liver or spleen cells (*210*). In adults with hookworm infection both the haemoglobin and serum ferritin concentrations are inversely correlated with the intensity of infection (*211*). These authors also found that serum ferritin was a valuable indicator of iron stores in populations infected with the helminths *Ascaris lumbricoides*, *Trichuris trichiura* and *Schistosoma mansoni*. To assess the value of iron status indicators in a population in which malnutrition, *Plasmodium falciparum* malaria and helminths were highly endemic, Stoltzfus et al. (*212*) examined the relationship between iron status indicators and erythropoiesis. The concentration of serum ferritin, erythrocyte protoporphyrin and sTfR were all significantly influenced by *Plasmodium* spp. infection, and the concentration of erythrocyte protoporphyrin and serum ferritin were also influenced by fever. The authors concluded that it would be "nearly impossible" to estimate the prevalence of iron deficiency in this population, except by a trial of therapeutic iron supplementation. A subsequent trial revealed that oral iron supplementation led to an increased concentration of SF and EP but had only a small effect on anaemia (*213*). The authors suggested that concurrent helminth infections may stimulate inflammatory immune responses in young children, with harmful effects on protein metabolism and erythropoiesis. Thus serum ferritin may be a valuable indicator of iron stores in some populations with chronic infection, but only after determining the infecting organisms and the relationship between the burden of infection and serum ferritin.

17.5 Detection of subjects with genetic haemochromatosis

Despite the first hopes that the serum ferritin assay would provide an effective way of detecting subjects with haemochromatosis early in their disease, it soon became clear that this was not the case. The first indication of iron accumulation is provided by measuring transferrin saturation (*181*) for reasons described above. The sensitivity and specificity of methods to detect nascent haemochromatosis can now be examined by studying subjects found to be homozygous for HFE C282Y, because 90% of patients with genetic haemochromatosis have this genotype in northern European populations (*214*). Beutler et al. (*215*) identified 152 subjects homozygous for C282Y among 41 038 individuals attending a clinic in California. Of these, 75% of men and 54% of women had a ferritin concentration greater than 250 µg/l and 200 µg/l respectively. Similar percentages had a raised transferrin saturation (75% and 40%). The mean age of these individuals was 57 years. In a prior analysis of the first 10 198 subjects (*189*) the sensitivity and specificity of serum ferritin and transferrin saturation to detect people with C282Y homozygosity was examined. Using optimal thresholds of ferritin >200 µg/l for women, ferritin >250 µg/l for men and a transferrin saturation of >45%, the sensitivities were 70% and 70%, and the specificities were 90% and 89% respectively.

In blood donors with a mean age of 38 years, Jackson et al. (*190*) identified 72 subjects who were homozygous for HFE C282Y from a total of 10 500 donors tested. Using

a transferrin saturation threshold of >50%, which was the 95th percentile of the value of first-time donors, the sensitivity of diagnosis was 45% and 86% for females and males respectively with a specificity of 97% and 95% respectively. Using a threshold serum ferritin concentration in women of >130 µg/l (the 95th percentile) the sensitivity of diagnosis was 22% ; for men a threshold of >210 µg/l gave a sensitivity of 34%. In both cases the specificity was high at 98%. The most powerful single diagnostic measure examined, an unsaturated iron binding capacity threshold of <20 µmol/l, showed a positive predictive value of 14% for men and 18% for women, with negative predictive values of >99.5% for both sexes. Although a combination of raised transferring saturation and serum ferritin concentrations gave a positive predictive value of 50% this was at the expense of sensitivity which only reached 22%. Of the 69 homozygotes for C282Y tested for iron status, only 15 had both a raised transferrin saturation and serum ferritin concentration.

18. Threshold values of ferritin to determine iron status

There are two approaches to establishing a threshold value of an indicator at which iron deficiency or iron overload is probable. The first is to identify subjects with iron deficiency or haemochromatosis. A threshold may be established from the range of values found in iron deficient or iron loaded patients. This approach has rarely been applied although the ferritin concentration during iron deficiency anaemia provides an example.

The second approach is to measure the concentration of the substance in healthy subjects not likely to be either iron deficient or iron loaded, and to calculate appropriate threshold values based on either 90 or 95% confidence intervals. This requires selecting subjects in order to exclude those with iron deficiency and possibly iron overload.

Thresholds for iron deficiency will be described for infants, children, adolescents and adults. In the case of iron overload, values are only available for adults.

18.1 Iron deficiency

Thresholds have been identified by examining the highest concentration found in patients with iron deficiency anaemia classified as microcytic, who have either an absence of stainable iron in their bone marrow or show a response to therapeutic iron (*1,148*). The suggested upper limit was 15 µg/l in each case. Table 9 shows thresholds derived from the analysis of iron replete populations. There are rapid changes in storage iron concentrations in the first 6 months of life. The WHO thresholds (4) appear to reflect results from clinical studies rather than an analysis of population distributions. The WHO report suggests that a ferritin concentration of <30 µg/l in children less than 5 years old in the presence of infection indicates depleted iron stores, but there is no consensus about this value in the literature. No threshold was suggested for adults with infection or inflammation.

18.2 Iron overload

The thresholds suggested for a serum ferritin concentration during iron overload have varied widely. WHO (*4*) concluded that thresholds of >200 µg/l for men and >150 µg/l for women were appropriate. In the UK values of >300 µg/l for men and elderly women, and >200 µg/l for young women, have been suggested (*216*). Thresholds

TABLE 9
Suggested thresholds for age groups to classify individuals as iron deficient during epidemiological studies

Age	Serum ferritin concentration (µg/l)	Reference
4 months	<20	Domellof et al. (*168*)[a]
6 months	<9	Domellof et al. (*168*)[a]
9 months	<5	Domellof et al. (*168*)[a]
1–2 years	<10	Looker et al. (*3*)[b]
3–5 years	<10	Looker et al. (*3*)[b]
<5 years	<12	WHO (*4*)
>5 years	<15	WHO (*4*)
6–11 years	<12	Looker et al. (*3*)
12–15 years	<12	Looker et al. (*3*)
<16 years	<12	Looker et al. (*3*)

[a] Data derived from studies of iron replete, breast-fed infants.
[b] Data from NHANES III.

from 200 µg/l (217) to 400 µg/l (*218*) have been applied to men. For pre-menopausal women 200 µg/l has been commonly selected as a threshold although Asberg et al. (*219*) used a value of >100 µg/l. Where good data are available age related thresholds should be applied (*47*).

19. Using ferritin to determine the iron status of populations

There have been two general approaches to assessing iron deficiency in populations. Iron deficiency has been defined as a serum ferritin concentration of <15 µg/l in studies conducted by Hallberg et al. (*148*) and Milman et al. (*207*) in Scandinavia. In the USA the criteria set to classify an iron deficiency in NHANES II and III have been two of three abnormal values for transferrin saturation, serum ferritin concentration and erythrocyte protoporphyrin concentration (*205*).

The first approach may over-estimate the frequency of iron deficiency. This is particularly critical in paediatric practice where the low concentration of serum ferritin is associated with a high coefficient of variation in the assay. The second approach tends to under-estimate the frequency, unless thresholds are carefully selected (e.g. a threshold at the 12th percentile for transferrin saturation instead of the 5th percentile for data from NHANES III).

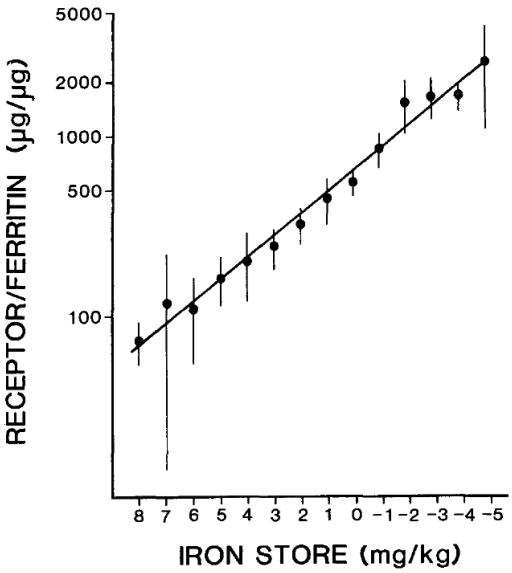
20. Using the ratio of serum transferrin receptor to serum ferritin to measure iron stores

Recently Cook et al. (*153*) have demonstrated that in healthy subjects the concentration of stored iron may be estimated from the ratio of sTfR/SF (reported in µg/l for both assays). The relationship between the sTfR/SF ratio and the concentration of stored iron was estimated in a study in which serial measurements of serum transferrin receptor and serum ferritin were made during repeated phlebotomy of 14 healthy subjects (*220*). There was a close, linear relationship between the logarithm of the sTfR/SF ratio and stored iron expressed as mg per kg body weight (Figure 4). The value of the ratio may be negative, which represents a deficit in iron required to maintain a normal haemoglobin concentration.

The equation of the regression line derived from this study has been applied to three situations: 1) estimating iron stores in the US population; 2) estimating changes in body iron after supplementation in pregnant Jamaican women; and 3) estimating changes in body iron during an iron fortification trial in anaemic Vietnamese women.

The results for people studied in the US population survey (a subset of NHANES III) confirmed a previous analysis (*221*), and the supplementation studies gave results similar to those found with earlier studies using radioactive iron or the "multiple measurement" approach. In the Viet Nam study the efficacy of the fortification strategy was demonstrated by studying only 30 subjects for 3 months. Fortification trials have previously required hundreds of participants and taken years to conduct (see references in (*153*)). The sTfR/SF ratio provided estimates of stored iron for each person in the survey, and their degree of anaemia due to iron deficiency could be calculated from the tissue iron deficit.

FIGURE 4
The variation in the serum transferrin receptor to serum ferritin concentration ratio with body iron. Positive values of body iron represent storage iron and negative values represent iron deficiency. Bars represent the standard error of the mean.



Adapted from Skikne et al. (*220*), with permission of the publisher.

Although a significant advance on previous methods, there are some qualifications: it will be difficult to validate the relationship between sTfR/SF ratio and body iron stores in children and pregnant women, two groups with the highest incidence of iron deficiency, by a similar process of quantitative phlebotomy, although non-invasive methods such as Superconducting Quantum Interference Device (SQUID) (*182*) may be applicable; and the ratio will be influenced by chronic disease because serum ferritin is one component of the ratio. The serum transferrin concentration is less influenced by infection than the ferritin concentration although there is debate about the reliability of measuring sTfR to estimate iron status during chronic disease and in populations where malaria is endemic. At present the only valid approach is to identify subjects with inflammation, infection or chronic disease and remove them from the analysis.

Currently the lack of standardization in assay ranges and units of measurement, and the lack of reference samples, prevents the wider use of the sTfR assay.

21. Recommendations for future surveys of iron status

There are four important principles:

1. Use the best possible approach based on current knowledge. Define anaemia based on the haemoglobin concentration. In principle, the serum ferritin concentration provides an index of the concentration of stored iron, but its use is limited because the ferritin concentration increases during acute and chronic disease. Neither erythrocyte protoporphyrin nor transferrin saturation provide a better index of stored iron concentration than serum ferritin concentration, although

they do identify iron-deficient erythropoiesis, but the value of TS is reduced by high biological variation. Rather than measuring lots of parameters to define iron deficiency, the sTfR/SF ratio should be further evaluated (see below).

2. An important feature of current work is to be able to compare results with previous studies and to detect trends. For the UK and Scandinavia this compatibility may require measurements of haemoglobin and serum ferritin in adults. For comparison with previous studies of infants and children in the USA (NHANES) and other international studies, transferrin saturation and zinc protoporphyrin concentration should be measured.
3. Standardized and reliable assays calibrated against reference material should be used.
4. Markers of inflammation or infection should be included. At the moment the reliable assessment of iron status is not possible in the presence of inflammation or infection, and it is necessary to exclude such subjects. The measurement of the concentration of C-reactive protein (CRP) provides indicator of acute disease whereas other proteins, such as alpha-1-acid glycoprotein (AGP), may provide a marker of chronic infection. An alternative is to measure the erythrocyte sedimentation rate (ESR). However none of these will identify minor infections that may increase the ferritin concentration for long periods (*63*) and a health questionnaire should be completed for each subject to identify possible infection. The use of highly sensitive assays for CRP may be valuable to detect sub-clinical infections. The ideal marker of infection has not been identified but ideally would have a similar response to disease as ferritin, but without being influenced by iron.

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ANNEX 3

Indicators of the iron status of populations: free erythrocyte protoporphyrin and zinc protoporphyrin; serum and plasma iron, total iron binding capacity and transferrin saturation; and serum transferrin receptor

JOHN BEARD

Contents

1. Introduction

The aim of this paper is to review and evaluate the state of knowledge regarding several indicators of iron status that may be used to assess the iron status of populations in developing countries. The indicators are: free erythrocyte protoporphyrin and zinc protoporphyrin; serum and plasma iron, total iron binding capacity and transferrin saturation; and serum transferrin receptor. In particular the epidemiological value of each variable will be considered and the technical limitations and advantages of each parameter when used in a public health setting.

2. Free erythrocyte protoporphyrin, erythrocyte protoporphyrin and zinc protoporphyrin

The conceptual basis for the measurement of protoporphyrin is a lack of iron in the bone marrow for incorporation into newly synthesized globin and the protein porphyrin as the haemoglobin molecule is reaching its final steps in synthesis. The last step in haemoglobin synthesis is the insertion of iron by the enzyme ferrochetalase. Instead of iron, trace amounts of zinc are incorporated into protoporphyrin instead. The normal ratio of iron to zinc in protoporphyrin is about 30 000:1, but a lack of iron available to ferrochetalase during the early stages of iron deficient erythropoiesis results in a measurable increase in the concentration of zinc protoporphyrin. When there is enough iron the reactions are as follows:

Reaction I:
Protoporphyrin + Fe++ → ferrous protoporphyrin + globin → haemoglobin

When there is a lack of iron then zinc replaces iron in a very small but measurable proportion of molecules:

Reaction II:
Protoporphyrin + Zn++ → zinc protoporphyrin + globin → ZPP-globin

Free erythrocyte protoporphyrin (FEP) is the compound left over after the zinc moiety has been removed using strong acids during the extraction and chemical measurement process. A variation of the chemical extraction method does not require this step and can provide direct measurements of ZPP and a very small amount of FEP. Typically FEP is less than 5% of the total. Thus FEP measurements, for all intents and purposes, are nearly identical to the ZPP measurements when the chemical extraction protocols are utilized. In turn, both FEP and ZPP should be interchangeable with the term "erythrocyte protoporphyrin" (EP).

The concentration of EP is expressed either as µg/dl of whole blood or µg/dl of red blood cells. The conversion of values relies on the accurate measurement of the packed cell volume.

A rise in the concentration of zinc protoporphyrin is one of the first indicators of insufficient iron in the bone marrow (*1*,*2*). The rate at which the concentration of EP rises in blood samples is proportional to the relative deficit in iron and the amount of erythropoiesis that is occurring. For example, reticulocytes typically contain more EP than mature red cells, so forms of haemolytic and aplastic anaemia which lead to reticulocytosis would be expected to have a higher concentration of EP than normal because the rate of erythropoiesis exceeds the supply of iron to the marrow. In uncomplicated iron deficiency, the concentration of EP is reported to increase within

1–2 weeks of a lack of iron in the bone marrow (3,4). After iron therapy begins, more than a month is required to re-establish a normal concentration of EP, and well after the restoration of normal plasma iron kinetics.

An increased concentration of EP and, in particular, the ratio of EP to haemoglobin (Hb), is an excellent indicator of a lack of iron to meet the normal demands of the bone marrow (*1*). The rates of reactions I and II above are such that a blood sample from a healthy normal individual will have an EP concentration of less than 40–50 µg EP/dl of red cells. Coincident with a fall in transferrin saturation below 15%, the concentration of EP increases rapidly to more than 70–100 µg/dl. With a prolonged or severe deficit in iron, the EP concentration may reach as high as 200 µg/dl.

Several stages in the human life cycle have large requirements for iron which the normal diet is unlikely to meet, including pregnancy and early infant development. In both of these stages of life the EP content of red blood cells has been examined with regard to its usefulness as a predictor of iron status.

2.1 Pregnancy

The large expansion in blood volume and red cell mass during the first half of pregnancy often depletes iron stores and leads to iron deficiency anaemia by the late-second or third trimester. An accurate diagnosis of iron status during all stages of pregnancy presents challenges (*5*) because both a dilution of blood volume and nutrient deficiencies can, and do, occur together. The recent paper by de Azevedo et al. (*6*) specifically examined the value of the several indicators of iron status during pregnancy. The authors demonstrated that folate and B_{12} deficiency were of moderate prevalence, and that the anaemia of pregnancy was not solely due to iron deficiency. The concentration of EP alone did not distinguish between women with iron deficiency and those without, because many women had an increased concentration of EP and those whose other measurements indicated iron deficiency, such as a raised serum transferrin concentration, were not predicted by a high EP concentration. Soluble transferrin receptor was the only indicator of iron status that appeared to be responsive to variations in iron status in this situation. Further evidence for a lack of sensitivity of EP to iron deficiency comes from a study that compared the concentrations of EP in mothers and their infants soon after birth, and observed little or no correlation (*7*).

2.2 Infancy

There has been a large emphasis on the use of EP in children because of its value in identifying children with lead poisoning, and because of the focus of many studies on this important period of growth and development. The ratio of EP/Hb is believed to be an accurate indicator of iron status in older children and adults (*1*,*2*), but its value in neonates is unclear. Erythropoiesis is very rapid in very young children which makes it difficult to determine a finite "normative" ratio or concentration. Several investigators have addressed this issue from the perspective of nutritional diagnosis. Infants less than 9 months of age with severe anaemia (Hb <50 g/l) had an EP/Hb ratio of >15 µg EP/g Hb while infants with less severe anaemia of Hb <90–100 g/l had an EP/Hb ratio of around 5–10 µg EP/g Hb (*8*). Thus, on initial examination, it appears that the EP/Hb ratio in infants can be used to diagnose iron deficiency even at young ages. More recently the EP/Hb ratio was evaluated in 143 infants in the first week of

life by giving iron supplements (*9*). The EP/Hb ratio was responsive to iron treatment in some, but not all, infants with a high ratio. This lack of certainty regarding a finite "ratio" that predicts iron deficiency forced the authors to conclude that EP in this age group was not a very useful means to identify individuals likely to respond to iron therapy (*9*). In a similar study of the utility of the ratio in slightly older infants who were born prematurely, Griffin et al. (*10*) concluded there was a correlation between EP and other measures of iron status after 6 months of life, but not before this age. Perhaps the study most pertinent to defining a proper threshold concentration of EP for children in their first year of life comes from the comparative studies of Domellof et al. (*11,12*). They examined by age and sex the differences in parameters of iron status measured in Swedish and Honduran breast-fed children less than 9 months old in an effort to better establish normative values in this age group. Boys had a lower mean cell volume (MCV) and concentrations of Hb and ferritin than girls, but had a higher concentration of ZPP and serum transferrin receptor (sTfR). When using their entire data set of 263 infants the authors estimated that the concentration of EP that was two standard deviations above the mean which was >70 µmol/mol for infants <6 months and >90 µmol/mol for infants >9 months old. In looking at children's response to treatment with iron the authors concluded that EP, Hb, and MCV were better predictors of iron status than sTfR and ferritin for infants <6 month old (*13*). A sensitivity analysis revealed that EP and MCV were more sensitive to iron status after controlling for confounding variables (*11*).

2.3 Childhood

The concentration of EP in children can easily rise to >200 µmol/mol during untreated iron deficiency anaemia; the rise in children with leukaemia and aplastic anaemia is not as large (>100 µmol/mol) and suggests that EP is sensitive to multiple causes of anaemia, and not just to iron deficiency (*13*).

In a study of children seen for general medical reasons, the ferritin concentration and EP/Hb ratio were equal in their ability to identify children who are iron deficient but otherwise apparently healthy (*14*). The children ranged in age from 8 months to 18 years and the EP/Hb ratio did not vary significantly with age or sex until the age of 13 years. A separate large sample of over 6 000 well nourished children was recently analyzed to generate normative values for boys and girls between birth and 18 years (*15*).

The data in Table 1 show that the age dependent threshold concentration of EP of >70 µg/dl red cells that is used to indicate iron deficiency in adults, the threshold of

TABLE 1
The threshold concentrations of erythrocyte protoporphyrin by age groups at which iron deficient erythropoiesis occurs according to Centers for Disease Control and Prevention (CDC) recommendations and the 97.5th percentile of the distribution of values in healthy subjects

Age	Recommended threshold[a] (µg/dl red cells)	97.5th percentile[b] (µg/dl red cells)
0–12 months	>80	40
1–2 years	>80	32
2–9 years	>70	30
10–17 years (girls)	>70	34

[a] CDC recommendation as the demarcation between iron deficient erythropoiesis and iron sufficient erythropoiesis (*16*).
[b] Adapted from Soldin et al. (*15*), with permission of the publisher.

> 80 µg/dl in 1–2 year old children, and the threshold of >75 µg/dl for 3–4 year olds, are much higher than the upper 97.5th percentile of values for healthy children. The practice of establishing thresholds based on the statistical distribution of a parameter would, in this recent study, have yielded very different thresholds from the values suggested by CDC for each age and sex group.

2.4 Response to iron therapy and complications

In simple and uncomplicated iron deficiency it is not uncommon to see the EP concentration drop within 4–8 weeks of intensive oral iron therapy: concentrations of >200 µmol/mol usually return to <60 µmol/mol within 2 months (*17*). In a study to estimate the sensitivity of iron status indicators, 62 patients who had provided bone marrow aspirates were studied (*18*). Reticulocyte production in response to iron therapy was correlated with iron stores in the bone marrow, but the concentration of ferritin, EP, and the sTfR to ferritin ratio, all had a significant predictive value to differentiate iron deficiency anaemia (IDA) from non IDA. Ferritin was the only significant and independent predictor of IDA in a multivariate analysis using a threshold concentration of 32 µg/l. The low diagnostic sensitivity and specificity of EP and the EP/Hb ratio meant that they were inadequate as single indicators of IDA in a group of anaemic patients and did not add to the predictive value of measuring the ferritin concentration. In contrast, Hastka et al. concluded from a small study of patients with chronic inflammatory diseases that EP can be used effectively to monitor the severity of the attenuated delivery of iron to marrow (*19*). Iron therapy does not always result in a decline in the concentration of EP if there is inflammatory or renal disease that blocks or attenuates the response of the bone marrow (*20*). The type, severity, and duration of this inflammatory effect seems likely to contribute to the lack of agreement regarding the usefulness of measuring the EP concentration in clinically complex situations.

A few papers have recorded a high ratio of EP/Hb in people with thalassaemia, but this is believed to be a result of the relative iron deficiency that occurs in this condition (*21*,*22*). In populations in which thalassaemia is a likely contributor to anaemia, measuring the concentration of EP may lead to a systematic bias (*23*).

The concentration of EP does not provide a specific indication of iron deficiency as it does not distinguish between the different reasons for a lack of iron in the bone marrow. A field trial was recently conducted in Côte d'Ivoire to assess if iron status was better predicted by the concentration of EP, sTfR, or ferritin in circumstances in which iron deficiency occurs concurrently with inflammatory disorders and infection (*24*). The investigators noted that the prevalence of iron deficiency was substantially higher if the concentration of EP was used to estimate the prevalence than if the ferritin concentration was used as the only indicator of iron deficiency. The authors adjusted the threshold for the concentration of ferritin upwards to 30 µg/l, to account for the fact that ferritin is an acute phase protein (*24*), and estimated the prevalence of iron deficiency using this criterion. The authors concluded that the sTfR concentration was a better indicator of depleted iron stores and of early iron deficiency in such a situation. It is not clear why the concentration of EP did not provide a similar estimate of the prevalence of iron deficiency as both of these indicators should be sensitive to the long term inflammation and infection caused by endemic malaria in this population.

2.5 Measurement, confounding factors, and units of measurement

Two general approaches are used to measure the concentration of erythrocyte protoporphyrin in blood samples. The older, chemical extraction method requires significant laboratory expertise and a sophisticated fluorescence spectrophotometer (*25*,*26*). The more popular and newer method requires only a dedicated hematofluorimeter, which measures reflective emissions from EP in a drop of blood.

Neither the chemical extraction method, nor the hematofluorimetric assay, require a fasting blood sample. This means that blood does not have to be collected at a particular time of day. A number of studies have demonstrated that both capillary and venous blood provide similar estimates. There is also not a need for any particular anticoagulant to be used unless long term storage of samples is desired (see below). However, both methods require that fresh blood samples should be used unless the blood is protected in some way from degradation by light of the fluorescent compounds. Blood samples collected on filter paper have been used successfully for several decades and provide a means to collect and transport samples collected in remote rural areas.

Porphyrin compounds fluoresce when exposed to light with a wavelength of 400–430 nm and the intensity is proportionate to the amount in a sample. By carefully selecting the emission wavelength only certain porphyrins are measured. The chemical method requires the extraction of the porphyrins in a solvent, protection of the samples from light, high quality external standards, and an appropriate spectrophotometer (*25*,*26*). Clearly, good laboratory practices are required to attain a coefficient of variation (CV) when analysing multiple samples of <5% (*14*). Fluorescence by haemoglobin can also be measured in the same way. These compounds are stable for up to 10 days if samples are protected from UV light and stored at 4 °C. An alternative is to collect the blood on filter paper in the field, dry it, store it in a sealed plastic bag, and then send the paper to a sophisticated laboratory where the concentration of EP can be measured.

Blood samples can be stored for prolonged periods of time at 4 °C and can be frozen for up to 3 months without a significant loss in EP content when acid-citrate-dextrose is used as an anticoagulant.

In contrast, a portable hematofluorimeter can be used to measure the amount of ZPP by reflective fluorescence, a method that requires only a drop of blood placed on a glass cover slip. The fluorescent material is ZPP while the absorbing compound at the excitation wavelength is haemoglobin. The porphyrin actually measured by this instrument is ZPP and the machine's manufacturer assumes that ZPP comprises 95% of all the erythrocyte protoporphyrin in blood, which provides the basis for estimating the concentration of EP. In some instruments the Hb concentration is also measured automatically at the same time and the output is reported as the ratio of EP/Hb as either µg EP/g Hb or as µmol EP/mol Hb (see below to convert from one unit to another). While calibration has been a problem for the first instruments manufactured, recent instruments appear to be more stable. Calibration is achieved by adjustments made at the factory, and by the frequent use of external and internal standards. It is possible to produce an internal standard from pooled samples of blood by washing it in sterile saline and storing a 1:1 volume ratio in citrate-buffered glycerol at -20 °C. This washed and cryo-protected blood can be used for up to 6 months as an internal control.

The field method of choice is clearly the hematofluorimeter as it requires only several minutes to do and <20 µl of capillary blood. The machine needs only a power

supply and requires little expertise to use. However the instrument does need calibration and makes two assumptions: that the mean corpuscular haemoglobin concentration (if expressed in µg/dl red cells) and the proportion of the total EP that contains zinc do not vary from sample to sample.

Two issues may affect measurements made using the hematofluorimeter: first, the need to oxygenate the blood sample; and second, the need to wash the red cells in saline before measurement. When fresh blood is taken either from capillaries or a vein, there appears to be little need to re-oxygenate the blood sample to get a reproducible measurement. However, when there is a considerable period of time between sampling and measurement (for example, greater that 30 minutes), there is better reproducibility when the blood is shaken or stirred gently to increase the oxygen content (*27*). This is less an issue with the narrower bandwidth filters put into instruments over the past 4–5 years than in the instruments produced in the 1980's and early 1990's.

There are several reports that washing red blood cells with sterile saline greatly lowers the number of falsely high results and improves the reproducibility and precision of the ZPP measurement, probably because it removes compounds from the serum that fluoresce at the same wavelength as the porphyrins (*17,27*). In these papers, there was a significant reduction in the concentration of ZPP in samples with high values by simply washing and re-suspending red cells in saline. This fact might account for some of the large variations in reported EP concentrations between studies. In clinical situations a high bilirubin concentration may contribute for this reason to falsely high ZPP concentration, but this is not likely to be a normal confounding factor variable in field trials.

The ability to measure the concentration of EP in a fluorescent reflective spectrophotometer allows the direct expression of EP as a ratio of ZPP/Hb because the haemoglobin concentration is also measured in the same machine in a separate photomultiplier tube and at a different wavelength. The actual concentration of erythrocyte porphyrin, FEP, EP, or ZPP, has been expressed as an amount relative to either whole blood, as µg/dl blood, or relative to the proportion of red blood cells as µg/dl red cells. If the haematocrit is known it is simple to convert µg EP/dl whole blood to µg/dl red cells as follows:

µg EP/dl red cells = µg EP/dl whole blood/Haematocrit

Or, if the ratio relative to the haemoglobin concentration is required:

µg EP/g Hb = µg EP/dl whole blood/g Hb/dl whole blood

To generally interconvert units (µg to µmol):

µg EP/dl red cells * 0.037 = µg EP/g Hb;
µg EP/dl red cells * 0.872 = µmol EP/mol Hb.

These latter two equations assume a normative value of the mean corpuscular haemoglobin concentration (MCHC); if it is possible actually to measure this, then there is no need to make this assumption.

The National Committee on clinical laboratory standards recommends the expression of amount of EP in a blood samples as a ratio of EP/Hb (*14*) in units of µmol EP/mol Hb.

CDC recommends the following thresholds to distinguish between iron deficient erythropoiesis and iron sufficient erythropoiesis :

- >70 µg EP/dl red cells (>2.6 µg/g Hb, >61 µmol/mol Hb) for children under the age of 5 years
- >80 µg EP/dl red cells (>3.0 µg/g Hb, >70 µmol/mol Hb) for individuals aged 5 years and above.

A number of studies have examined the sensitivity and specificity of using different thresholds of EP concentration as a means of screening for iron deficiency anaemia. The best thresholds that optimize sensitivity and specificity are discussed by Mei et al. (*28*). It is worth noting that one clinical study used a threshold of 55 µmol/mol to define a high concentration of ZPP based on 2 standard deviations (SD) above a mean concentration of 35 µmol/mol (*7*). In this case the threshold was successfully used to screen potential blood donors and thus avoid the need to defer them.

The day-to-day variation in the concentration of EP is around 6.5% (*29*) and is considerably more than reported by the same authors for concentrations of serum iron and ferritin, at 3.0% and 3.7% respectively. There are some data on the coefficient of variation using the older chemical extraction method, although very good reproducibility can be attained by laboratories accustomed to performing the assay (*26*).

The biological source of the day-to-day variations is not clear since one would expect it to be similar to the variation in haemoglobin concentration or in the mean cell haemoglobin concentration, as these are formed elements within red cells and not subject to large daily fluctuations in concentration. The CV for Hb is reported to be <2% in some studies (*30*).

A document from the International Nutritional Anemia Consultative Group (INACG) on measurements of iron status notes that the concentration of EP measured using a haematofluorimeter is systematically 10–20% lower than if measured using the extraction method (*25*). Instruments that assume a fixed packed red cell volume of 35% when expressing the concentration of ZPP per dl of red cells will tend to overestimate the concentration.

Lead interferes with the synthesis of haem by inhibiting porphobilinogen synthase which results in an increased urinary excretion of ALA (aminolaevulinic acid). There may also be a direct effect of lead on the activity of the enzyme ferrochetalase which leads to an increase in the amount of metal-free protoporphyrin and results in an increase in the need for iron to insert into the porphyrin ring structure. The competition between zinc and iron at the level of the ferrochetalase is shifted toward the production of zinc protoporphyrin in contrast to iron protoporphyrin (*31*,*32*). The rise in EP or ZPP concentration that results from this shift reflects disruption in the incorporation of iron into protoporphyrin due to lead, and is not due to a dietary iron deficiency. The concentration of lead in the blood necessary to be a health risk is >10 µg/dl, according to 1991 CDC guidelines, but is not reproducibly and specifically related to an increase in concentration of ZPP (31). Nonetheless, Labbe et al. suggest that ZPP can be an effective screening test for lead exposure in adults (*1*). There are 14 toxicology reports in just the last 3 years on the relationship between blood lead concentrations due either to acute or chronic exposure, and alterations in the concentration of ZPP, Hb, and other indices of haemoglobin production (see reference 2 for a review). In general these are reports of very high concentrations of lead in blood and are consistent with the 1999 report from Israel in which more than 14% of 105 workers had blood lead concentrations of >60 µg/dl and which were correlated with the concentration of ZPP (*33*). However, a thorough statistical analysis was not done in many of these reports so that it is not possible to identify the threshold for an effect.

Labbe and Dewanji recently reviewed and reiterated their view that ZPP is a cost-effective screening test that can identify individuals with nutritional and non-nutritional causes of iron deficient erythropoiesis (*2*). Since EP is not sensitive to acute inflammation and it is not time consuming or expensive to measure the concentration, there is some strong appeal for its use in screening people. Table 2 shows Labbe and Dewanji's suggested thresholds and their interpretation.

TABLE 2
The concentrations and ratios of indicators of iron status, their interpretation and recommendations

Measurements	Interpretation	Recommendation
Low ZPP/Hb ratio (<60 µmol/mol)	Adequate systemic iron supply	Iron stores can be estimated by the ferritin concentration; If ZPP/Hb ratio <40 µmol/mol, consider tests for iron overload (TSAT)
Mid-range ZPP/Hb ratio (60–80 µmol/mol)	Possible non-replete iron status; consider inadequate diet, ACD, or other causes	CBC may support case for iron depletion; ferritin can be used to differentiate low iron stores from inflammatory blockade; could then use concentration to verify inflammation
High ZPP/Hb ratio, (>80 µmol/mol) and low ferritin concentration (<20 µg/l)	Iron deficient erythropoiesis attributable to low marrow iron supply, maybe to depleted iron stores	Iron supplementation; monitor therapy with decrease in ratio and/or increase in reticulocyte count
High ZPP/Hb ratio (>80 µmol/mol) and high ferritin concentration ; (>200 µg/l)	Severe inflammatory blockade, ACD, other causes of impaired iron utilization	Correct the causes(s) of impaired iron utilization; consider chronic lead poisoning or ineffective supplementation

ZPP, zinc protoporphyrin; Hb, haemoglobin; CBC, complete blood count; ACD, anaemia of chronic disease; APP, acute phase protein; TSAT, transferrin saturation.
Adapted from Labbe et al. (*1*), with permission of the publisher.

While the values given in Table 2 may not be a perfect approach for use in field studies in developing countries, it does offer a method to evaluate the value of EP measurements to screen populations.

Mei and colleagues (*28*) recently published a careful analysis of the relative sensitivity and specificity of the concentration of Hb and EP in children and adult women to diagnose iron deficiency. A receiver operating characteristics (ROC) analysis of iron status indicators demonstrated that the concentration of EP was quite a sensitive diagnostic indicator for children, but not for adult women. The reasons for this difference was not immediately obvious as the data set did not focus on very young (<12 months) children for whom there are known problems regarding the interpretation of EP concentrations.

The approach suggested by Labbe et al. (*1*) needs to be applied to a number of large data sets to assess the usefulness of the concentration EP or the EP/Hb ratio to diagnose iron deficiency and to decide whether a combination of these variables with ferritin or sTfR concentration can be used in public health screening and surveillance programs as well as in research studies.

3. Serum iron, total iron binding capacity and transferrin saturation

3.1 Serum iron

The plasma or serum pool of iron is that fraction of all iron in the body that circulates bound primarily to transferrin. There are usually enough binding sites on transferrin so that 100% saturation does not occur, and the typical range in saturation is 35–

45% of all binding sites. The iron in this pool turns over very quickly and represents iron in transit from one location to another e.g. from absorptive cells to erythrocytes developing in the bone marrow. The iron bound to low molecular weight proteins such as dicarboxylic acids is not usually detected in most assays although there are methods such as ultrafiltration, for example, that allow an estimate of the concentration of non-transferrin bound iron in the plasma pool. This usually comprises less than 1% of the total plasma iron pool, but there are situations in which it can increase substantially.

There are three ways of assessing the amount of iron in the plasma or serum: by measuring the total iron content per unit volume in µg/dl; by measuring the total number of binding sites for iron atoms on transferrin, called the total iron binding capacity, in µg/dl; and by estimating the percentage of the two bindings sites on all transferrin molecules that are occupied, called the percentage transferrin saturation.

More than 80% of the iron in plasma is taken up by developing erythroblasts in the bone marrow. This means that changes in the rate of red cell synthesis alters both the rate of turnover of plasma iron and the concentration of iron in plasma. The clinical conditions that are associated with a rapid release of iron into the plasma pool from surrounding tissue and cells, such as reperfusion injury, repeated transfusions, and prematurity, can exceed the iron binding capacity of transferrin. Measured in terms of plasma turnover, iron exists in the plasma pool for a short period, typically 40–50 minutes. This means that the concentration of iron in plasma, or serum, changes quickly with the very dynamic movement of iron from tissue (e.g. enterocytes, reticuloendothelial cells, hepatocytes, others) into the plasma pool as well as the movement of iron out of the plasma pool into tissue (e.g. bone marrow, myocytes, blood brain barrier, etc.). Most of the iron entering the plasma pool is derived from iron recycled from catabolised red blood cells in the reticulo-endothelial system. The release of iron from macrophages, such as during responses to cytokines in acute inflammation, will result in substantial changes in the plasma iron concentration.

Two significant biological factors that alter the plasma iron concentration are infection and inflammation. The plasma iron concentration is very responsive to cytokines released from immune cells throughout the inflammatory process. The cytokines interleukin-6, interleukin-2, interleukin-10, and tumour necrosis factor α (TNF-α) are all potent stimuli for the movement of iron from the plasma pool into storage sites in macrophages. The rate of "normalization" of this acute phase response varies between individuals which adds uncertainty to the interpretation of plasma iron in populations and individuals experiencing inflammatory processes. For example, the plasma iron concentration may return to normal within 24–48 hours of an acute infection but may remain low for prolonged periods of time during chronic inflammatory states such as arthritis. This results in a decreased availability of iron to cells leading to the anaemia of chronic disease (ACD), a condition often seen in elderly people.

Finally, after a meal a significant rise in plasma iron concentration can be observed due to the release of iron absorbed by enterocytes into the plasma pool. The measurement of the plasma iron concentration during this period will be representative of the absorption of iron.

The normal plasma iron concentration ranges between 50–120 µg/dl (*25*,*34*). The thresholds used to classify individuals as iron deficient typically range from 50–

60 µg/dl, however natural variation in measurements may lead to misclassification (*34,35*). There is substantial day-to-day variation within subjects of approximately 15%, as well as variation during the day of 10–20% (*30,35*). The variation between subjects is at least as large and suggests that assessing iron status based on a single measurement of plasma iron concentration has a high risk of misclassifying iron status and may lead to an error in any estimate of prevalence.

The amount of iron in plasma can be readily measured by a number of methods that are quantitatively reliable, sensitive, reproducible, and require very small amounts of sample (*25,34*). Determinations of plasma or serum iron are based on either colorimetric principles or are made by direct measurement using an instrument such as an atomic absorption spectrophotometer. These methods have been well described elsewhere and have been fundamentally unchanged for more than two decades (*25,34*). There are some needs that must be addressed in order to get reliable results, regardless of the assay:

- Care must be taken to prevent contamination of samples during collection with iron from needles and plastic-ware;
- Standardized blood sampling protocols must be used, since the time of day that the samples are collected and post-prandial effects can be quite pronounced.
- Laboratory techniques must be used that minimize contamination with iron from equipment and the environment;
- A high level of competency is required to ensure good laboratory practices and reproducible results.

3.2 Transferrin, total iron binding capacity, transferrin saturation

The globular protein, transferrin, is the specific transport protein for iron in the plasma pool, and each molecule binds with similar affinity two molecules of iron. The protein circulates throughout the plasma pool and delivers iron to cells via the transferrin receptor pathway (*34*). The concentration of transferrin increases during iron deficiency and decreases with protein deficiency, so it is sensitive to several factors. The concentration of this transport protein reflects iron status only when iron stores are exhausted and when the plasma iron concentration is <40–60 µg/dl, so it does not diagnose iron deficiency prior to ineffective erythropoiesis. A proxy measure of transferrin is the measurement of the total iron binding capacity (TIBC) which applies the assay for plasma iron with one additional step to measure the iron saturation of transferrin.

Because the concentration of transferrin is often estimated by measuring the total iron binding capacity, it is susceptible to the same problems as measuring the serum iron concentration.

The assay is really a measure of the total number of transferrin binding sites per unit volume of plasma or serum and is performed much like the serum iron assay. The TIBC is not as subject to rapid changes in concentration as the plasma iron concentration, so it is inherently more stable as an indicator of iron status. The TIBC by itself is not often used as a measure of iron status because it appears not to change until iron stores are depleted (*34*).

Clinical studies have demonstrated that a transferrin saturation (TSAT) of <15% is insufficient to meet normal daily requirements for erythropoiesis (*34*). A prolonged period of time with a TSAT below 15% results in iron deficient erythropoiesis which

leads to changes in the number and shape of newly released reticulocytes and erythrocytes (*36*).

The variation in concentration of iron in plasma and serum iron is substantial, as described above, and this variation causes the daily variation in TSAT (*5*). The TIBC itself shows a very little variation either within subjects or from day to day. Anything that alters the plasma iron concentration will alter the TSAT, thus there is the same lack of specificity for TSAT as there is for plasma iron concentration.

The concentration of transferrin in plasma or serum can be readily measured using a variety of immunological methods such as enzyme-linked immunosorbent assays (ELISA) or immunoblotting, both using commercially available monoclonal or polyclonal antibodies. The assays themselves are usually "sandwich" ELISA methods, similar to the assay used to estimate the concentration of ferritin, or they use a single antibody to transferrin. These assays use antibodies to specific epitopes on the circulating glycoprotein and allow the precise measurements of concentrations of transferrin in plasma and serum (*34*). In contrast to colorimetric methods and to atomic absorption spectrophotometry, there is less need to prevent contamination with iron when using an immunological assay. The limitations of such assays may be significant. First, the antibodies used in different assays may vary in their affinity for transferrin epitopes and may bind to different absolute amounts of transferrin during assays. Second, there is no internationally used external standard, so comparisons between different assays are difficult to make. Finally, most laboratories fail to perform reliability tests between ELISA plates. This is a very important source of variability and can be an important source of error in the determining the concentration of transferrin.

The range in the coefficient of variation of TIBC within an individual from day-to-day is about 8–12% while the diurnal variation is less than 5% (*29*,*30*,*35*). Assay variation is rarely reported though it is quite low in laboratories which use well established methods (*25*,*29*,*30*).

4. Soluble transferrin receptor

The measurement of soluble transferrin receptor (sTfR) has become popular in the past 10 years because it is sensitive to the inadequate delivery of iron to bone marrow and tissue (*37*). During cellular iron deficiency the concentration of sTfR increases in plasma or serum and is not strongly affected by concurrent infection. The sTfR concentration can be measured in a quantitatively reproducible manner, it is reasonably stable in concentration within an individual, and can be interpreted as an indicator of the severity of abnormal erythropoiesis.

The sTfR fragment is cleaved from the membrane bound transferrin receptor found on nearly all cells. The predominant donors of these fragments to the plasma or serum pool are the cells of the developing red cell mass – the erythroblasts and reticulocytes. The concentration of sTfR reflects erythropoietic activity. The concentration decreases in situations and individuals with marrow hypoplasia, such as after chemotherapy for cancer, while the concentration increases in individuals with stimulated erythropoiesis, such as haemolytic anaemia and sickle cell anaemia. Since sTfR is sensitive to erythropoiesis due to any cause, it cannot be interpreted strictly as an indicator of iron deficiency erythropoiesis. This means that sTfR is an indicator of iron status only when iron stores are empty and there are no other known causes of abnormal erythropoiesis (*37*–*39*).

Most of the assays for sTfR that have been developed to date are based on immunological methods to identify the amount of cleaved protein fragment in circulation in the plasma pool. The development of an ELISA more than 10 years ago (*37,40,41*) and the quantification of the relationship between iron deficiency anaemia and the sTfR concentration led to the commercial production of several assay kits. As mentioned before, such assays are not easy to do well. The commercial kits require an ELISA plate reader with a narrow bandwidth detection beam, and a familiarity with antibody based assays is essential. A stable internal standard is absolutely necessary as there is no external standard currently in existence. This means that assays are difficult to compare. The diurnal variation in concentration of sTfR is poorly described but is believed to be <5%. However, this is not well established with regard to the time of day as morning to evening variation has not been differentiated from the day to night variation. The variation within and between assays is fairly small when good techniques are employed. For example, Cooper and Zlotkin reported a methodological variation of 5–6% during a study of a large cohort of people in Canada (*42*). In the same study the day to day variation in ferritin was between 12–24%, and there was nearly twice as much variation in females compared with males. Similar degrees of variation are reported by manufacturers of commercial assay kits. Run-to-run variation in assay performance needs to be monitored to prevent drift in the assay than cannot easily be detected without an external standard. Differences between different lots of antibodies should also be assessed, especially if the antibody is not commercially produced.

Finally, because the concentration of sTfR is not normally distributed, values may need to be transformed to logarithms before analysis (*43,44*).

The absence of an international standard to allow different assays to be compared has made it difficult to define accurately a specific range of normal values. Beguin recently published normal values of 5 ± 1 mg/l (*39*). A popular commercial kit in the United States uses a somewhat higher threshold of 6–7 mg/l. It is impossible to assign a single threshold value that would be accurate for all commercial kits and clinical instruments. During severe beta thalassaemia the concentration of sTfR is typically >100 mg/l while during severe iron deficiency anaemia it is >20–30 mg/l.

A number of factors may affect the concentration of sTfR in plasma or sera: acute or chronic inflammation and the anaemia of chronic disease, malaria, malnutrition, age and pregnancy.

There is conflicting evidence regarding the impact of inflammation and cytokines on the sTfR concentration. Feelders et al. (*45*) showed that administering TNF-α to cancer patients caused a prompt decrease in the concentration of sTfR that persisted for a period that was distinct from the time course response for other acute phase proteins such as ferritin, C-reactive protein and acid glycoprotein. During chronic inflammation, such as during the ACD, Beguin states that it is possible to have a normal TfR despite an increase in ZPP concentration in some patients with ACD (38). This suggests a depletion in bone marrow iron content without a measurable increase in the concentration of sTfR (*38*). Others have demonstrated that if iron deficiency anaemia and ACD coexist, then the sTfR concentration can increase (*46*), but this has not been reported in all studies (*47*). The sTfR/log ferritin index has been found to be useful to separate individuals with IDA and ACD (*47*). Yet the gold standard to asses iron status is a bone marrow biopsy for iron and, when this was used by Junca et al. (*48*), they failed to find that the sTfR concentration predicted a decrease

in the amount of iron in bone marrow in patients with ACD. Follow up studies of 129 anaemic hospital patients again used bone marrow aspirates to identify an iron deficiency and observed an increase in sTfR concentration in IDA, but not in ACD (*49*). The sTfR log ferritin index was again able to separate subjects with IDA from those with ACD among people with both conditions. In a smaller study, Pettersson et al. (50) showed that the concentration of sTfR was decreased during ACD, and was increased during IDA and in subjects with a combination of IDA and ACD.

Malaria is associated with sudden changes in measures of iron status because of the cyclical effect of disease on red cell metabolism. Two potential effects on the concentration of sTfR may be expected: (*37*) the increased concentration of cytokines attenuates the concentration of erythropoietin leading to a reduction in erythropoiesis and an associated decrease in sTfR concentration; (*38*) active haemolysis of red blood cells leads to anaemia with an associated increase in the concentrations of erythropoietin and sTfR. Both effects may occur in populations where malaria is endemic.

Verhoef et al. (*43*) recently showed that the concentration of sTfR was increased in infants with both IDA and malaria in Africa. There was however, a significantly greater increase in sTfR concentration in anaemic children with malaria than in anaemic children without malaria. As expected in children with malaria, there was a very substantial variation in the sTfR concentration which reflected the cyclical nature of the disease. Other studies of a similar size and scope showed a similar effect of malaria on the sTfR concentration (*44,51*) although it was not always possible to conclude that the sTfR concentration was increased independently of iron deficiency anaemia. These data suggest that it is difficult to determine the iron status of people with malaria using sTfR.

A study reported by Kuvibidila et al. (*52*) found no effect of moderate protein energy malnutrition on the sTfR concentration of African women with a wide range in haemoglobin concentration.

Because the concentration of sTfR changes during erythropoiesis, infants should have a higher sTfR concentration than adults (*53*). Yeung and Zlotkin (*54*) noted that the concentration of sTfR did not vary by age or sex in infants aged 6–15 month old and that all the infants had a higher sTfR concentration than is usually observed in adults. But the lack of normative data for young children prevents a clear cut diagnostic use of sTfR concentration for detecting iron deficiency in this age group.

The relationship between an increased sTfR concentration and depleted iron stores, a low transferrin saturation, a low plasma iron concentration, and the anaemia that is characteristic of iron deficient erythropoiesis in adults, is hard to confirm in young children. For example, two studies have found an expected inverse correlation between the concentration of sTfR and Hb, iron, and TIBC in infants (55,56), while a third study found no relationship between sTfR and measurements of iron status in newborns (*57*).

The complicated situation in children with beta thalassaemia has been examined (*56*). A high concentration of sTfR was noted in the newborn children of mothers that were iron deficient, but could not use sTfR to distinguish between children with beta thalassaemia and those who were iron deficient. In contrast to patients with ACD mentioned earlier, the sTfR/ferritin ratio, or the log ferritin index could not distinguish between these two conditions either.

Pregnancy is another physiological situation characterized by very active erythropoiesis. Akinsooto et al. (*58*) used the concentrations of ferritin and sTfR in pregnant women in an attempt to find out which had the better sensitivity and specificity to

detect iron deficiency as diagnosed by other indicators. The sTfR concentration and the sTfR/ferritin index were 75% and 86% sensitive, and 63% and 82% specific, respectively. The positive predictive values were 64% and 84%, and the negative predictive values were 75% and 87% for iron deficiency. Studies by Akesson et al. (*4*) and Carriaga et al. (*59*) found the sTfR concentration to be useful to diagnose iron deficiency during pregnancy, and achieved nearly 100% specificity, yet other studies have not found this to be the case. In one of the few studies to use bone marrow aspirates, Van den Broek et al. (*60*) concluded that the sTfR concentration was not as good a predictor of iron status as ferritin concentration in pregnant Malawian women.

The studies of Cook et al. (*37*) provided direct evidence that the sTfR concentration rises within several weeks of repeated phlebotomy, once iron stores have been depleted. Furthermore, the sTfR concentration was not a useful indicator until the ferritin concentration was <20 µg/l. These data indicate the capacity of sTfR measurements to detect a rapid fall in iron stores. Others have asked the opposite question: how long does it take to detect a significant reduction in sTfR concentration when iron therapy is given? Souminen et al. (*46*) gave oral iron supplements to 65 individuals who were frequent blood donors and examined changes in sTfR concentration. There was a significant decrease in sTfR concentration within 3 months of supplementation. It is likely that studies that look for changes in sTfR concentration over shorter periods will demonstrate that 6–8 weeks is sufficient to detect a significant biological change of >0.5 SD units.

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ANNEX 4

The interpretation of indicators of iron status during an acute phase response

CHRISTINE A. NORTHROP-CLEWES

Contents

1. Abstract

Iron status is influenced by infection or trauma. The objective of this paper is to describe how indices of iron status, particularly the concentration of ferritin and soluble transferrin receptor (TfR), are influenced by changes in the concentration of acute phase proteins (APPs) during infection or trauma. Measurements of the change in concentration of APPs after elective surgery that are not preceded by infection are used to show the difference in responses of these proteins. Changes in the concentration of APPs and markers of iron status during treatment for infection are also used to demonstrate the interrelationship between indicators. In developing countries asymptomatic malaria is common and produces an acute phase response, but the data on the concentration of APPs and indices of iron status in asymptomatic malaria are inconclusive with respect to using either serum TfR or ferritin as a marker of iron status in such situations. In individuals infected with human immunodeficiency virus (HIV) there may be an atypical acute phase response in the absence of opportunistic infections. Some tentative conclusions are drawn concerning the interrelationships between ferritin and two APPs, C-reactive protein (CRP) and α-1-acid glycoprotein (AGP), during an acute phase response.

2. Introduction

Ferritin concentration is an important indicator of total body iron stores and a concentration of <12–15 µg/l is taken to indicate deficient iron stores (*1*). However, the synthesis of ferritin is stimulated by infection, which may either obscure an iron deficiency or indicate a larger iron store than truly exists. In this paper, the nature of the acute phase response and its relationship to iron metabolism is illustrated.

Iron deficiency is the most common micronutrient deficiency in the world. It can affect all populations and age groups, but the most vulnerable groups are women and children. Anaemia is commonly used as an indicator of iron deficiency in population-based surveys, but iron deficiency is not the only cause of anaemia: infections, haemoglobinopathies, and vitamin A deficiency can all lead to anaemia. A high prevalence of anaemia is often found in developing countries, especially where infections such as malaria or hookworm are common. In addition infection with HIV is affecting millions of people in the developing world and may influence their iron status, but little is known about the acute phase response during HIV infection in the absence of opportunistic infection.

3. The acute phase response

Infection and trauma are accompanied by an acute phase response, a non-specific process that includes the production of APPs prior to the full activation of the immune response. The main purpose of the acute phase response is to prevent damage to tissues, and remove harmful molecules and pathogens. During such a response the concentration of some APPs, called positive APPs, increase in the plasma and others, called negative APPs, decrease. The changes in the concentrations of APPs are due largely to changes in their production by hepatocytes, which in turn are regulated by cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α), which act in a complex network (*2*).

The role of the positive APPs includes host-adaptive and host-defense mecha-

nisms, which act by binding to foreign substances and by modulating phagocytic cell functions. The positive APPs include CRP, α-1-antichymotrypsin (ACT), AGP, also known as orosomucoid, serum amyloid A (SAA), fibrinogen, haptoglobin, caeruloplasmin and ferritin. An increase in serum ferritin concentration occurs in response to any infectious or inflammatory process, but serum ferritin concentration also reflects total body iron stores, hence a low serum ferritin concentration can only reflect depleted iron stores in the absence of infection. The magnitude of the change in concentration of the APPs during an acute phase response varies considerably: caeruloplasmin can increase by about 50% whereas CRP can increase by as much as 1000-fold (*3*,*4*). The APPs that decrease in concentration include transferrin, albumin, transthyretin and retinol binding protein (RBP). These proteins are not thought to have an immune function, but rather to act as transport proteins and as a result, the plasma concentration of the specific nutrients they carry may be reduced during infection and inflammation (*4*).

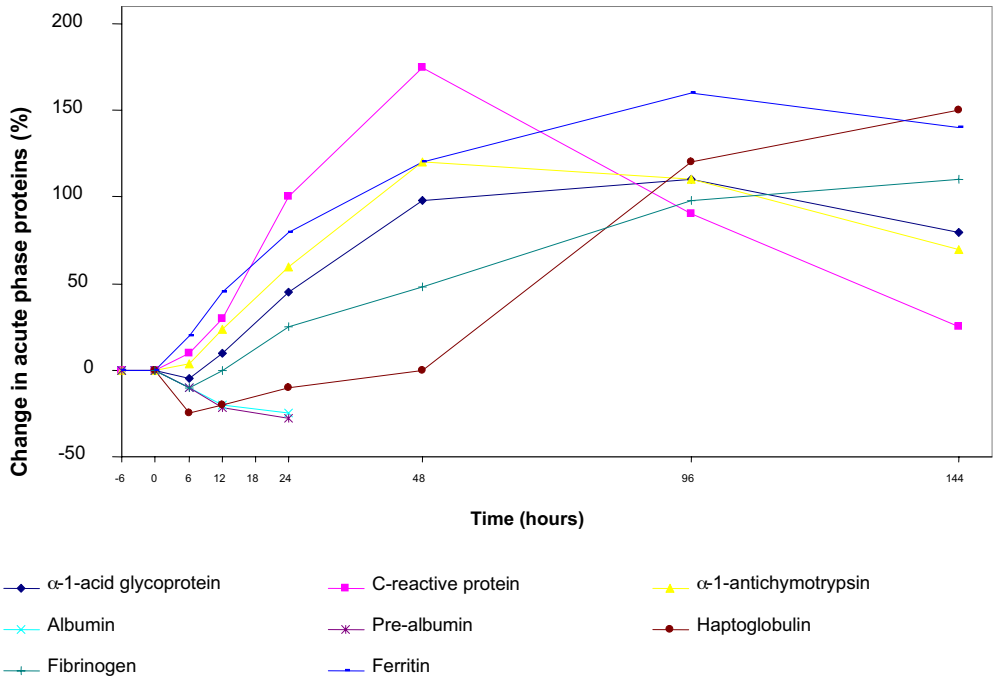
4. Sequence of events

The concentration of APPs after elective surgery that is not preceded by infection can be used to demonstrate the time course over which their production is stimulated. The first change during the acute phase response is in cortisol concentration, which peaks at 6 hours after surgery, followed by a rise in the number of white blood cells, which peak at 10 hours. Data obtained after cholecystectomy indicate that CRP and ACT rise rapidly in the first 6 hours and continue to rise for the first 20 hours after surgery, whereas AGP shows only a small increase during the same time period (Figure 1). Albumin and pre-albumin both decrease in concentration. Haptoglobulin initially decreases but returns to its initial concentration by 20 hours before showing a steady increase in concentration over the next few days (*4*).

Data collected over 6 days following elective surgery showed that CRP rises very rapidly from normal concentrations of <5 mg/l to concentrations of >100 mg/l, peaking at day 2, and then slowly decline. Fibrinogen and AGP show a slower initial rise but do not return to a normal concentration until up to a week after CRP had returned to normal (*4*). Data from Fleck and Myers (*4*), however, showed that the rise in concentration of AGP and fibrinogen was preceded by a slight fall in concentration immediately after the operation. An initial decrease in AGP was also observed by Myers et al. (*5*) 2–4 hours after making a skin incision for a hysterectomy, and this preceded an increase in AGP at 6 hours. The initial decrease in AGP concentration is thought to be due to changes in microvascular permeability immediately following trauma which may facilitate the movement of proteins between the plasma and damaged tissues (*3*).

Data on the concentration of CRP, SAA, haptoglobulin, fibrinogen, and albumin collected over a period of 21 days after surgery confirmed and extended the information from the first study (*4*). Both CRP and SAA increased rapidly and reached a peak concentration between 48–72 hours after surgery. With no post-operative complications, the concentration of CRP returned to normal by the 10th post-operative day, whereas SAA did not return to normal values until about 12–14 days after the operation. In contrast, haptoglobulin and fibrinogen initially decreased in concentration, then slowly increased, and only achieved maximum concentrations at 10 and 12 days, respectively; fibrinogen returned to normal, but the concentration of haptoglobulin was still higher than normal at day 21 (*3*).

FIGURE 1
Characteristic patterns of change in acute phase proteins (APPs) after trauma. APPs are shown as percent change from the initial concentration.



Adapted from Feelders et al. (*2*), Gabay and Kushner (*3*), Fleck and Myers (*4*), Wieringa et al. (*6*), with permission of the publishers.

5. Changes in acute phase indicators during treatments for various diseases

Baynes et al. (*7*) reported a number of small studies in which they followed the changes in concentration of various acute phase indices in patients with different illnesses, and showed sequential changes during treatment.

The increase in plasma ferritin concentration paralleled the increase in plasma CRP during acute pneumonia, tuberculosis, rheumatoid arthritis and neutropenic sepsis, suggesting that ferritin was acting as an APP. However, the degree to which the ferritin concentration rose was influenced by the underlying iron status of the subjects. The plasma ferritin concentration was found to be much lower in individuals with rheumatoid arthritis, who were iron deficient as well as being chronically ill, whereas the concentration of CRP always seemed to be increased in proportion to the severity of disease.

In neutropenic sepsis, both CRP and ferritin were markedly increased in concentration. During treatment there was a sharp drop in CRP concentration that was not paralleled by a drop in plasma ferritin concentration. The different responses may reflect different turnover rates, with CRP concentration responding rapidly to the removal of the trauma (i.e. the chemotherapy), whereas the ferritin concentration declined more slowly, perhaps because erythropoiesis was still depressed by the production of cytokines (*7*).

6. Acute phase proteins, iron indicators and the influence of cytokines

A low serum iron concentration and a high serum ferritin concentration during an acute phase response are associated with a redistribution of iron into the liver and mononuclear phagocyte system, both mediated by cytokines (*2*). In experimental animals the administration of the cytokines TNF-α, IL-1 and IL-6 induced a decrease in serum iron concentration within 3–6 hours and in similar studies *in vitro*, cytokines induced an increase in ferritin synthesis (*2*). Feelders et al. (*2*) carried out an experiment in humans and reported similar results to those found *in vitro* and in experimental animals, i.e. the administration of cytokines resulted in hypoferraemia associated with an increased ferritin production.

In the study by Feelders et al. (*2*) 12 patients with inoperable soft-tissue sarcoma or melanoma were treated for 2 days with the T-cell-derived cytokine human interferon γ (IFN γ), which is thought to prime activated macrophages. Serum samples were taken on two days before treatment and at baseline. All patients were then cannulated, an isolated limb perfusion was performed using recombinant human TNF-α and human IFN γ, and further blood samples were taken for 7 days after treatment. Although an acute phase response occurs in many types of cancer (*8*,*9*) and would be expected to produce associated changes in iron metabolism, Feelders et al. (*2*) showed that APPs (except the concentration of AGP, which was slightly higher than normal) and iron status were normal before treatment, suggesting that the local tumours were not producing a systemic reaction.

Following treatment with recombinant TNF-α and IFN γ, all APPs except CRP and ferritin decreased in concentration, probably due to haemodilution and capillary leakage. Both CRP and ferritin increased in concentration after the start of perfusion, representing an early acute phase response, while AGP responded more slowly in a second response. The concentration of CRP showed a sharp decrease after peaking at day 2; ferritin decreased slowly in concentration but was still higher than normal at day 7, as was AGP. In contrast the two negative APPs, albumin and transferrin, decreased in concentration from the first pre-treatment day, reached a nadir at the baseline sample, then remained low for 2 more days before slowly increasing in concentration. Serum iron and serum TfR concentrations decreased during pretreatment, and remained low after perfusion, with the lowest concentrations recorded 8 hours and 1 day afterwards, respectively.

The experiment showed that the administration of TNF in humans caused hypoferraemia associated with an increased ferritin production. The data also confirm a previous report that the increase in ferritin concentrations parallels that of CRP (*7*), suggesting that ferritin responds as an early APP. However the plasma ferritin concentration remained high for longer than the CRP concentration thus, in the latter part of the experiment, ferritin behaved more like AGP.

7. Control of iron metabolism

The regulation of iron metabolism is normally under the control of iron regulatory proteins (IRPs) that bind to sequences on messenger ribonucleic acid (mRNA) and protect mRNA from degradation. As a consequence of iron deficiency the IRPs bind to mRNA which promotes the expression of transferrin receptor protein and represses the synthesis of ferritin. When iron is present in adequate amounts, ferritin syn-

thesis is promoted and iron storage occurs. During infection the normal control of iron metabolism is changed by IL-1 and TNF-α. The plasma ferritin concentration increases, despite a low concentration of serum iron, because ferritin mRNA is more sensitive to cytokines than to iron (*10*).

The low concentration of serum iron found during infection (hypoferraemia) is accompanied by changes in the plasma concentration of several iron-binding proteins (Table 1) that facilitate iron uptake by the reticuloendothelial system of the gut or the removal and re-use of haemoglobin released from old erythrocytes (*10*).

TABLE 1
Influence of inflammation on iron-binding proteins in plasma

Plasma protein (units)	Normal range	Function	Change in plasma concentration in response to infection
Caeruloplasmin (g/l)	0.16–0.53	Converts Fe^{II} to Fe^{III}	Increases ~ 50%
Transferrin (g/l) (μmol/l)	1.9–2.58 25–34	Binds and transports iron	Decreases ~ 30%
Lactoferrin (μg/l)	0.91–0.45	Binds iron, especially at low pH	Released from granulocytes. Increases 200–500%
Ferritin (μg/l) (pmol/l)	15–250 34–562	Binds iron	Can increase 3000%
Haptoglobulin (g/l) (μmol/l)	0.70 – 3.79 7.0–37.9	Binds haemoglobin	Increases 200–500%

Adapted from Thurnham and Northrop-Clewes (*10*), with permission of the publisher.

Hypoferraemia may protect individuals against infection by withholding iron from pathogenic micro-organisms and by reducing the potential pro-oxidant properties of iron which may exacerbate tissue damage at the site of inflammation where reactive oxygen species are being produced and the body's cells are at risk of damage (*11*). One theory suggested that lactoferrin acts in the hypoferraemic-hyperferritinaemic response to inflammation by causing a drop in plasma iron concentration and a rise in plasma ferritin by removing iron from transferrin and delivering it to the macrophages, where it is bound to ferritin (*11*). However, it is now thought that lactoferrins exert antimicrobial properties that are independent of binding iron, although their mode of action has not been elucidated (*10*).

8. Ferritin

In clinical practice the gold standard to estimate iron stores is to stain a bone marrow aspirate for iron, but this is not practical to do during population surveys, so alternative methods have been sought. The World Health Organization recommends that a serum ferritin concentration <12 μg/l indicates depleted iron stores in children <5 years of age, while a concentration <15 μg/l indicates depleted iron stores in those >5 years of age (*1*). However, both thresholds may be too low during an acute phase response or when there is chronic disease, and a serum ferritin concentration between 30 and 100 μg/l may better indicate depleted iron stores in such circumstances (*1*,*12*).

The fact that the measurement of serum ferritin concentration can reflect the total body iron store and an acute phase response has been known since the 1970s, but the exact kinetics of the changes that occur are not known in detail (*2*). Understanding how to interpret the concentration of serum ferritin in the presence of infection

is difficult, and various approaches have been suggested. An early idea was to use the ferritin concentration and the mean corpuscular volume or haematocrit, which is low during anaemia, but it can also be altered by a wide variety of conditions including B vitamin deficiencies, liver, thyroid and kidney disease, and so was not a good choice. It became clear that to interpret the serum ferritin concentration some measure of the acute phase response was needed (*12*). Therefore it was suggested that, in addition to ferritin, an independent indicator of the acute phase response, such as CRP or AGP, should be measured (*13*). The report of the joint World Health Organization/Centers of Disease Control and Prevention technical consultation on the assessment of iron status at the population level proposes a meta-analysis of existing data to explore the possibility of using one or two APPs to correct serum ferritin concentration in the presence of infection.

Other approaches to help interpret the ferritin concentration in the presence of infection include: the determination of serum iron concentration with the percentage saturation of serum iron-binding capacity (transferrin), because a high serum ferritin concentration and a transferrin saturation <45% usually indicates infection (14); the measurement of TfR alone (discussed below) because it is thought to be unaffected by infection; and the measurement of TfR plus ferritin. However there is disagreement about the use of ferritin as well as TfR because it is thought that ferritin does not improve the diagnostic efficiency of measuring TfR alone, or that the calculation of the TfR/log ferritin ratio is more useful (*15*).

9. Serum transferrin receptor

Measuring the concentration of serum TfR is as an alternative method to assess iron status because the concentration increases during iron deficiency. It is thought that the serum TfR concentration is not increased in individuals during an acute phase response therefore the measurement of serum TfR may help to distinguish between individuals with and without iron deficiency in the presence of infection. However no international reference standard exists for this assay, and there may be difficulties in interpreting serum TfR in the presence of some chronic infections (*15*).

Transferrin is the main iron transport protein found in blood. It delivers iron to cells where it interacts with the specific membrane receptor, called TfR (*14*). Serum TfR is a truncated monomer of the tissue receptor, lacking the first 100 amino acids (*15*). Mean serum TfR concentrations tend to be in the range of 5–8 mg/l in normal subjects, but standards for different commercial assays vary and results cannot easily be compared.

Serum TfR concentrations can range from 8 times below to 20 times above normal values. The most important factor controlling this variation is bone marrow erythropoietic activity (*15*). Serum TfR concentrations indicate the absolute rate of erythropoiesis and the adequacy of marrow proliferative capacity for any level of anaemia. As the iron supply to the tissues becomes deficient the concentration of TfR on cell surfaces increases progressively and independently of the presence of adequate iron stores. This means that an increase in serum TfR concentration is a sensitive and quick response to the development of iron deficiency. Conversely, the serum TfR concentration decreases in response to treatment with iron before a change in haemoglobin occurs, so the response to iron can be monitored by changes in serum TfR (*15*).

The serum TfR concentration may be slightly increased to 9 mg/l in non-anaemic iron deficiency, but can be much higher (25 mg/l), in iron deficiency anaemia (IDA). The concentration of serum TfR is not increased if there is an acute phase response (Table 2) therefore serum TfR distinguishes between IDA and iron deficiency due to an acute phase response. The absence of an increase in TfR concentration during the anaemia of chronic disease (ACD) is due to the action of the cytokine IL-1, the primary mediator of the acute phase response. During ACD erythrocyte survival is somewhat reduced and IL-1 prevents the adequate release of iron from the reticuloendothelial stores so there is not enough iron for erythropoiesis. In conditions where there is ACD as well as IDA, then the serum TfR concentration may be increased to a similar degree as for IDA alone (Table 2).

TABLE 2
Differential diagnosis of iron deficiency anaemia (IDA), anaemia of chronic disease (ACD) and the combination of both, where N indicates no change in the concentration or ratios of indicators and the arrows indicate the direction of a change.

	Concentrations of:				Ratio of soluble transferrin receptor/ ferritin
	Haemoglobin	Serum iron	Ferritin	Soluble transferrin receptor	
Iron deficiency anaemia (IDA)	↓	↓	↓	↑	↑ ↑
Anaemia of chronic disease (ACD)	↓	↓	N-↓	N	N
ACD and IDA	↓	↓	↓	↑	↑

Adapted from Beguin (*15*), with permission of the publisher.

Unfortunately the serum TfR concentration may not always distinguish between patients with or without iron deficiency in the presence of some chronic conditions. For example, in some forms of ACD, the serum TfR concentration may remain normal even when IDA is present, because marrow erythropoietic activity may be suppressed by cytokines. Therefore the relationship between iron status and the serum TfR concentration in inflammation may be affected by the degree of anaemia but also, and more importantly, by the effect of the cytokines on erythropoietic activity. It has been proposed that the combined use of the concentration of serum TfR and ferritin, or the use of the ratio of the concentration of serum TfR/ferritin or serum TfR/log ferritin, may help to identify iron deficiency in patients with a chronic acute phase reaction (*16*,*17*). In particular, Beguin (*15*) suggests that the log (serum TfR/ ferritin) ratio may prove to be the most useful.

10. Asymptomatic malaria, acute phase proteins and iron

Assessing iron status in areas where malaria is endemic presents difficulties in symptomatic and apparently healthy individuals. Continual exposure to malaria parasites induces varying degrees of immunity in a population, such that asymptomatic and apparently healthy adults and older children have parasitaemia but no clinical disease. The presence of parasites may produce a chronic acute phase response, even in an asymptomatic individual, resulting in an elevated serum ferritin concentration. Asymptomatic malaria is also associated with a lower than normal haemoglobin concentration and an increased serum TfR concentration, which indicates that the presence of the parasite is associated with haemolysis.

Several studies support the finding of a raised serum TfR concentration in people

with asymptomatic malaria (*18–20*). However Stoltzfus et al. (18) found that although the serum TfR increased with parasite density in children, this increase disappeared when serum TfR was adjusted for the haemoglobin concentration. Furthermore, Verhoef et al. (*21*) suggest that, because of malaria-induced haemolysis, the serum TfR concentration may not be a useful measure of iron deficiency in individuals with malaria, and that further studies are needed to elucidate the relationship between the serum TfR concentration and malaria.

Stoltzfus et al. (*18*) found no relationship between the serum ferritin concentration and the density of malaria parasites when the concentration of parasites was <1000/µl blood, but above this parasite density the serum ferritin concentration was higher by 1.5 µg/l per 1000 parasites. Odunukwe et al. (22) have proposed that serum ferritin could be a useful marker of iron status during asymptomatic malaria. They suggested that there is a linear relationship between the serum ferritin concentration and malaria parasite density in apparently healthy adults, irrespective of the species of *Plasmodium*, and that the ferritin concentration can be corrected using the following formula:

Measured serum ferritin concentration – (0.08 µg * malaria density) = ferritin level (µg/l), where malaria density is measured in counts/µl blood.

The usefulness of this formula has not yet been confirmed.

11. Human immunodeficiency virus, acute phase proteins and iron

Infection with HIV is accompanied by a progressive accumulation of iron in macrophages, endothelial and other cells, and can result in an iron excess in the bone marrow, brain and other organs during the advanced stages of disease. The main cause of the iron excess is the chronic acute phase response which retains iron in the reticulo-endothlial system and acts to sustain a low serum iron concentration (*23*). A consequence of such iron loading is the growth of microorganisms, resulting in the opportunistic infections typical of acquired immunodeficiency syndrome (AIDS).

Because iron is needed for lymphocyte activation and proliferation, an altered immune function related to imbalances of iron metabolism might be a special problem in patients with HIV (*24*). The proliferative phase of lymphocyte activation requires iron because it is essential for enzymes such as ribonucleotide reductase, which is involved in deoxyribonucleic acid (DNA) synthesis (*24*). Changes in iron status appear to exert subtle effects on the immune system in HIV by altering the proliferation of T-lymphocytes and B-lymphocytes (*25*).

The results of a number of studies suggest that infection by HIV alone can elicit an APP response but, overall, the acute phase response in asymptomatic HIV is mild (*26–30*). In a study of children with HIV infection, without secondary infection, Jahoor et al. (*29*) found that the APP response may be different from the response elicited by bacterial infections because the higher concentrations and faster synthesis rates of the positive APPs were not accompanied by lower concentrations and synthesis rates of the negative APPs. The results from this study of children (*29*) confirmed previous data from the same author on asymptomatic HIV-infected adults. Their findings suggest that because the negative APPs are also transport proteins, the concentration of the specific nutrients they carry may not be reduced during early but asymptomatic HIV infection.

12. Summary

- An increased concentration of serum TfR is a good indicator of tissue iron deficiency, irrespective of iron stores.
- The concentration of serum TfR is not increased when anaemia is due to inflammation, so changes in the serum TfR concentration can distinguish between IDA and ACD. However, when IDA and ACD are both present, the serum TfR concentration is increased, irrespective of the acute phase response.
- In some chronic conditions erythropoietic activity may be increased even though there is no IDA and, in such circumstances, the concentration of serumTfR may be difficult to interpret. Various ways of using the ratio of serum TfR to serum ferritin have been suggested to help identify IDA in the presence of such a chronic acute phase response.
- Normally the synthesis of ferritin and TfR proteins is regulated post-transcriptionally by the intracellular iron concentration. However, an increased ferritin synthesis during an acute phase response appears to be stimulated by cytokines, independently of intracellular iron.
- Serum ferritin increases during an acute phase response, although the final concentration of ferritin is influenced by the underlying iron status.
- In the studies cited, ferritin behaved as a fast-acting positive APP at the beginning of the acute phase response and paralleled the changes in concentration of CRP. However in the later stages of the acute phase response, the concentration of ferritin remained high, and it behaved more like AGP.
- Thus, in the initial phase of infection the change in concentration of CRP may predict the behaviour of ferritin, and in the later stages AGP may control for the confounding effects of the acute phase response. Measurement of both these APPs may help to interpret the changes in serum ferritin concentration: if only the CRP concentration is elevated then the infection is in the initial stages; if both CRP and AGP are elevated in concentration then the infection is in the acute stage; and if only AGP is elevated then the infection is in the chronic stage and a correction factor to interpret ferritin in each stage could be calculated.
- More information is needed to interpret iron status as a result of an acute phase response. The report from the joint World Health Organization/Centers for Disease Control and Prevention technical consultation on the assessment of iron status at the population level suggests the use of a meta-analysis to explore the possibilities of using one or two APPs to correct ferritin in the presence of infection.

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