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

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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.

5. References

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