Assessment and interpretation of micronutrient status during pregnancy

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Accurate assessment of maternal micronutrient status is critical to the prevention of suboptimal micronutrient status and anaemia during pregnancy. Measurement of Fe, folate and vitamin B12 status is complicated by adaptive changes to maternal and placental physiology that markedly affect concentrations of circulating micronutrients and their functional biomarkers. Validation of new assessment methods by comparison with gold standards is often prevented by ethical considerations. Antenatal screening in the UK is predominantly concerned with the detection of anaemia, although estimation of maternal Fe stores by serum ferritin at the start of antenatal care may be a more effective preventive strategy. Functional assessment of maternal anaemia is highly problematic, so instead reference data are used for its definition. The effect of mild-to-moderate anaemia on pregnancy outcome is unclear because of the crude nature of its assessment and the influence of confounding factors. Fe-deficient erythropoiesis may be detected by assessment of erythrocyte Zn protoporphyrin and reticulocyte Hb, although such measures may be unavailable in many clinical laboratories. Serum soluble transferrin receptor is highly responsive to tissue Fe deficiency and is less affected by inflammation than most other indicators. Direct inter-assay comparison of serum and erythrocyte folate values is inadvisable since recovery rates differ greatly between methods. Serum total homocysteine is a useful functional biomarker of both folate and vitamin B12 status but during pregnancy is influenced by other factors that reduce its sensitivity. Isotope-dilution liquid chromatography–tandem MS and serum holo-transcobalamin provide new opportunities to gain detailed data of folate species and vitamin B12 fractions in large samples.

Micronutrient status: Pregnancy: Assessment methods: Maternal anaemia

Adequate micronutrient nutrition is essential during pregnancy to ensure optimal fetal growth(1). Whilst a wide range of micronutrient deficiencies is commonly found in developing countries(2), concern in countries such as the UK is focused on a few micronutrients such as folate, vitamin B12, Fe and vitamin D(3–6). Pregnancy is a time of huge changes in maternal physiology, several of which may influence micronutrient concentrations and biomarkers to an extent not seen in non-pregnant non-lactating (NPNL) women(7,8). Ethical constraints often prevent indicators of micronutrient status from being rigorously assessed in pregnant women because of the invasive nature of most gold standard methods. In the absence of specific validation studies there remains a danger that normal assessment methods and reference ranges will be assumed to be valid and that interpretation will not account for the adaptive maternal, placental and fetal responses to increased micronutrient demands during pregnancy. The present review discusses the assessment of Fe, folate, and vitamin B12 status in pregnant women and describes the methods used to screen routinely for maternal anaemia caused by prolonged deficiency of any of these micronutrients.

Abbreviations: CHr, reticulocyte Hb content; IDA, Fe-deficiency anaemia; IDE, Fe-deficient erythropoiesis; MCV, mean corpuscular volume; NPNL, non-pregnant non-lactating; RCF, erythrocyte folate; sTfR, soluble transferrin receptor, TC, transcobalamin; tHcy, total homocysteine; ZnPP, zinc protoporphyrin.

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Routine antenatal screening for anaemia caused by micronutrient deficiency

Anaemia can be defined as an insufficient erythrocyte mass to deliver adequate amounts of O₂ to peripheral tissues⁹. Maintenance of the erythrocyte mass is limited by the ability of maturing erythroblasts to acquire sufficient amounts of Fe, folate and vitamin B₁₂ for the synthesis of DNA and haem¹⁰. These micronutrients can be mobilised from body stores, absorbed from the diet or recycled from senescent erythrocytes¹⁰,¹¹. During pregnancy the demand for micronutrients, especially Fe and folate, is greatly increased and maternal body stores and dietary intake may be insufficient to meet demand. Inadequate supply of these micronutrients to the bone marrow results in dysfunctional erythropoiesis and ultimately anaemia.

In many industrialised countries routine antenatal care involves screening for anaemia at the initial booking appointment, normally between 10 weeks and 20 weeks of gestation, and at 28 weeks of gestation¹². In the UK screening relies heavily on the full blood count, an array of haematological indices that provides information on the number, size and Hb content of the erythrocyte population. An abnormal result or suspicion of increased risk justifies the use of more precise methods to identify underlying problems. Most patients, however, are assessed using only full blood count data.

Mean corpuscular volume: the multi-purpose screening tool

The mean corpuscular volume (MCV) is used to screen for folate, vitamin B₁₂ and Fe deficiency and is measured as part of the full blood count. Poor folate or vitamin B₁₂ status increases MCV by impairing DNA synthesis and cell division, whereas Fe deficiency decreases MCV as a result of impaired Hb production⁹. The normal range in NPNL adult women is 80–100 fl, although during pregnancy this level is elevated slightly because of the physiological increase in the proportion of younger larger erythrocytes⁸,¹³. When screening for Fe-deficiency anaemia (IDA), MCV is considered alongside Hb and packed cell volume.

The detection of anaemia by MCV tends to occur only after functional deficiency lasting several weeks or more, since it takes time to repopulate the erythrocyte mass with enough cells of abnormal size to affect the mean markedly. Sensitivity is further reduced during pregnancy since the physiological increase in MCV can potentially mask the early stages of microcytosis⁸. It is therefore most useful at the initial booking appointment since it can identify pre-existing micronutrient deficiency that can be treated in time to prevent further deterioration¹⁴–¹⁶. During late pregnancy, however, it may fail to detect individuals with recently-developed deficiency brought about by the high demand for micronutrients¹⁷,¹⁸.

Hb: the maternal oxygen-carrying capacity

The conceptus is totally dependent on the maternal supply of O₂ for its own respiration and this supply is delivered to the placenta, bound to Hb, at a rate governed by several factors including cardiac output, ventilation rate, uterine blood flow, smoking, altitude and packed cell volume¹⁹. Insufficient supply of Hb to the placenta caused by maternal anaemia can potentially result in fetal hypoxia with serious adverse consequences for fetal development²⁰.

Impaired Hb production during pregnancy is most often caused by poor Fe status, and therefore measurement of Hb serves as a useful screening method for the detection of IDA. Assessment is complicated by the physiological expansion of the plasma volume during early pregnancy and the subsequent increase in erythrocyte mass²¹,²². The net result of these haematological changes is a 5–15% dilution of the maternal blood supply, which reduces blood viscosity and improves substrate delivery to the placental²³,²⁴. This process is called the 'physiological anaemia of pregnancy' and does not reflect any deterioration in maternal Fe status or O₂-carrying capacity.

There is considerable debate as to what constitutes the optimal Hb range during pregnancy. The US Centre for Disease Control has classified maternal anaemia based on data from healthy Fe-supplemented mostly-Scandinavian pregnant women²⁵–²⁸. Using the 5th percentiles from these groups to define the limit of the normal range, the Centre for Disease Control has defined anaemia as Hb <110 g/l during the 1st and 3rd trimesters and <105 g/l during the 2nd trimester. Similarly-derived cut-off points have also been established for packed cell volumes. A more recent dataset of several hundred healthy pregnant Danish women has found similar results. Normal ranges (g/l), defined as means±1.96 SD, were: 118 (105–132) at 18 weeks of gestation, 118 (103–134) at 32 weeks of gestation, and 124 (108–140) at 39 weeks of gestation⁸. These values are assumed to represent a healthy range of values within these populations, since no clinically-significant adverse pregnancy outcomes were recorded. Hb concentrations below these lower cut-off points are considered likely to reflect maternal IDA to some extent.

The effects of uncomplicated IDA in human pregnancy are difficult to ascertain since ethical considerations often prevent controlled studies from being carried out. One alternative is to observe the relationships between IDA and pregnancy outcomes in free-living populations. Unfortunately, such studies are confounded by factors such as inadequate haemodilution²⁹,³⁰, smoking³¹, chronic inflammation³², infection³³,³⁴ and low BMI³⁴, all of which have strong independent effects on pregnancy outcome. Most studies have reported the optimal Hb range in mid-to-late pregnancy to include mild anaemia as defined by Centre for Disease Control values (Hb 95–110 g/l)³⁵–³⁸ and a few have even questioned the risks associated with moderate or severe IDA (Hb <95 g/l and <80 g/l respectively), since the numerous observational studies that have examined this issue are highly inconsistent²³.

An alternative method is to examine the effects of maternal anaemia in highly-controlled animal models. Most of these studies have used ovine models, although in studies that have been carried out in human subjects using non-invasive techniques the results have generally been in agreement²⁰,³⁹,⁴⁰. These results show adaptive responses by both mother and fetus to acute falls in maternal packed...
cell volume that help to prevent maternal and fetal hypoxia. The affinity of Hb for O₂ is reduced by increasing erythrocyte concentrations of 2,3-diphosphoglycerate, which allows more efficient uptake of O₂ by maternal and fetal tissue⁴⁹,⁵¹. Increased maternal cardiac output and utero–placental blood flow maintains the rate of O₂ delivery⁴⁰,⁴⁶ and increased fetal heart rate and blood pressure maintains the distribution of O₂ to fetal tissue⁴². In addition, both mother and fetus may reduce their rates of physical activity to levels that require less O₂. Only when O₂ delivery falls to below 50% of normal levels, or when conditions persist for several days, does the rate of fetal O₂ consumption fall as a linear function of O₂ delivery⁴³,⁴⁴. Under such conditions the fetus adapts to the lower supply of O₂ by up regulating erythropoiesis and down regulating fetal growth, both of which help to prevent the onset of hypoxia or acidosis in all but the most extreme conditions.

Whilst the effects of mild maternal anaemia remain highly controversial, moderate-to-severe anaemia in both animal models and human observational studies has been consistently associated with fetal growth restriction, probably mediated by chronically-impaired maternal O₂-carrying capacity⁴⁰,⁴¹,⁴⁵,⁴⁶. However, it remains unclear whether there is any discernable benefit of Fe therapy in terms of pregnancy outcomes, other than restoration of maternal Fe status⁵⁵. The continuing practice of prophylactic Fe supplementation is based on the general consensus that Fe deficiency is by definition undesirable and that its prevention is more likely to be beneficial than harmful⁴⁷. Maternal supplementation during pregnancy is also likely to improve neonatal Fe status and thus protect the infant from IDA, especially if born preterm⁴⁷–⁴⁹.

Assessment of maternal body iron stores

Body Fe stores are located mostly in the reticuloendothelial cells of the bone marrow, liver and spleen, as well as in hepatocytes⁵⁰. The largest pool of Fe is contained in the maternal erythrocyte mass, which is constantly turned over and recycled. The most precise methods for the assessment of body Fe stores are quantitative phlebotomy⁵¹, liver biopsy⁵², MRI⁵³ and bone marrow staining⁵⁴. However, the routine use of such techniques during pregnancy would be both unethical and expensive.

Fortunately, body Fe stores can be measured quite accurately using serum ferritin. Ferritin serves as the body’s main intracellular Fe storage protein. Trace amounts of mostly Fe-free ferritin are secreted into the plasma from reticuloendothelial or parenchymal cells in concentrations that linearly reflect total body Fe stores⁵⁵. In NPNL women a serum ferritin concentration of 1 µg/l is equivalent to approximately 7–8 mg mobilisable Fe⁵⁵. Thus, a serum ferritin concentration of 50 µg/l indicates adequate Fe stores (350–400 mg) for the day-to-day needs of most NPNL women. However, as >500 mg Fe stores is required to prevent depletion of Fe stores during the average pregnancy⁵⁷, only serum ferritin concentrations of >70–80 µg/l in early pregnancy are likely to reflect sufficient Fe stores able to prevent depletion without the need for supplementation⁵⁸,⁵⁹.

The interpretation of serum ferritin is based not on reference data from healthy individuals, as with the detection of IDA, but rather on the concentrations at which depleted body Fe stores can be predicted with great confidence. In NPNL individuals a serum ferritin of <15–16 µg/l identifies the absence of stainable bone marrow with 75% sensitivity and 98% specificity⁶⁰,⁶¹. Only one study has carried out similar assessments of pregnant women⁶². In this study the most accurate cut-off point in late pregnancy was determined to be 30 µg/l, which is markedly higher than that found in NPNL women. Sensitivity and specificity at this level are 90.0% and 85.1% respectively, compared with 37.5% and 93.7% for 12 µg/l; data for the commonly-used cut-off at 15 µg/l were not presented.

The higher cut-off point found in this study may have been a result of the high levels of inflammation in the sample. Pregnancy is associated with a physiological increase in inflammatory biomarkers, especially during the 1st and 3rd trimesters⁶³,⁶⁴. Serum ferritin becomes elevated during inflammation because of its role as an acute-phase reactant, and therefore may overestimate body Fe stores⁶⁵. High serum ferritin can also result from damage to ferritin-rich tissues that releases it into the maternal circulation, such as can occur in cases of pre-eclampsia⁶⁶. Inflammation and infection tend to affect the entire serum ferritin distribution, not just those individuals with high concentrations⁶⁷ and therefore, whenever possible, serum ferritin should be measured alongside inflammatory markers such as C-reactive protein, α1-antichymotrypsin, α1-acid glycoprotein or erythrocyte sedimentation rate⁶⁸,⁶⁹.

The relationship between serum ferritin and pregnancy outcome is confounded by several of the same factors that complicate the assessment of anaemia. Maternal infection, inflammation and hypertensive disorders are all associated with a higher incidence of preterm delivery and fetal growth restriction⁷⁰–⁷³. Thus, whilst it appears from observational studies that serum ferritin concentrations <30 µg/l during the second half of pregnancy are protective⁶², this is most probably because the risks associated with these confounders are much greater than those associated with depletion of maternal Fe stores. It is unlikely that there are any beneficial effects of maternal Fe stores depletion per se, since healthy women supplemented with Fe from early pregnancy experience no increase in adverse pregnancy outcomes and are less likely to become Fe deficient⁷⁴.

Assessment of iron-deficient erythrocytosis

Fe-deficient erythrocytosis (IDE) is the synthesis of erythrocytes deficient in Hb as a result of a lack of available Fe. The main difference between IDE and IDA is that IDE reflects current erythrocyte production whereas IDA reflects the state of the erythrocyte population as a whole. As only 1–2% of the erythrocyte mass is replaced each day, it can take several weeks of IDE before its effects become clinically apparent in the form of anaemia.
Conversely, once effective Fe therapy begins IDE will cease yet IDA can persist for several weeks until a substantial proportion of the erythrocyte population is replaced by normochromic normocytic cells (75). Although the reduced O2-carrying capacity caused by IDE may be compensated by an array of adaptive responses, depletion of tissue Fe has widespread, if not clinically-apparent, effects on the maternal metabolism. Reductions in cellular Fe concentrations may adversely affect the function of a wide range of Fe-dependent tissue enzymes such as catalases, peroxidases and cytochromes (76,77). The early detection and treatment of IDE is therefore important not only to prevent the onset of IDA, but also to ensure optimal Fe-dependent metabolism throughout pregnancy.

**Erythrocyte zinc protoporphyrin**

The most established biomarker of IDE is zinc protoporphyrin (ZnPP), which measures the extent to which Zn, rather than Fe, has been chelated with protoporphyrin. Increasing amounts of ZnPP are produced in maturing erythroblasts as Fe availability becomes suboptimal in the bone marrow. This process may function as a homeostatic mechanism by inhibiting the excretion of Fe following haemolysis by macrophages (78). Thus, it is a highly-sensitive functional indicator of IDE, especially when presented as ZnPP:haem, as this variable controls for haemodilution during pregnancy (79). ZnPP remains constant throughout pregnancy in Fe-replete women, whereas in unsupplemented women it tends to rise markedly in the last trimester (80,81).

Although ZnPP measures IDE with reasonable accuracy, it cannot distinguish between true and functional Fe deficiency. The latter occurs when body Fe stores are adequate yet Fe is not available to the bone marrow, such as can happen during infection and inflammation. The underlying cause of IDE may be identified by concurrent assessment of serum ferritin. For instance, if both measures are high then IDE caused by chronic inflammation is more likely, whereas if ZnPP is high (≥60 μmol/mol haem) and serum ferritin is low (<15 μg/l) then true IDE caused by depleted Fe stores is more likely. This combined use of ZnPP and ferritin values has been recommended by several authors as a reliable method for the determination of IDE (82,83). However, it should be noted that functional and true IDE are not mutually exclusive processes and may co-exist, especially during late pregnancy when low-level inflammation with depleted Fe stores is a common occurrence. In such circumstances the relative contributions of the underlying causes of IDE may be difficult to quantify.

The main advantages of ZnPP are its cost efficiency and its ability to be used in the field by unskilled personnel using a portable haematofluorimeter. However, its use is limited by its high sensitivity to environmental Pb pollution (84), which inhibits the binding of Fe to protoporphyrin, and its poor sensitivity in identifying improvements once Fe therapy has begun. This limitation might be resolved if the assay could be refined to measure only reticulocyte ZnPP, as has been suggested by some authors, and initial crude efforts to this effect have shown considerable promise (85). Despite its advantages, however, the lack of an automated method for assessment of ZnPP has severely constrained its use in most clinical laboratories.

**Reticulocyte indices**

Reticulocytes are immature erythrocyte cells that under normal conditions comprise about 1% of the erythrocyte population. They mature in the bone marrow for 1–3 d and are then released in the maternal circulation for 1–2 d before they lose their RNA and become fully mature. Reticulocyte counts have been available in most clinical laboratories for many years as a measure of the rate of erythropoiesis. However, a few modern haematological analysers now have the ability to measure the Hb content of reticulocytes, which effectively measures the extent of IDE over the previous 3–4 d (86).

Reticulocyte Hb content (CHr) is similar in some aspects to ZnPP. It is a cellular measure of IDE that on its own does not distinguish between true and functional Fe deficiency. However, unlike ZnPP, analysis is restricted to reticulocytes, so its sensitivity to short-term changes in Hb production is much greater (87). Values >26–28 pg identify IDE in NPNL adults (88).

CHr works by assessment of the reticulocyte cell volume and Hb content; hence conditions that change MCV independently of Fe deficiency will lower the precision of CHr (89). Such conditions include microcytosis caused by thalassaemia and macrocytosis caused by folate or vitamin B12 deficiency (90). Unfortunately, concurrent Fe and folate deficiency is relatively common during late pregnancy (91). Whilst initial validation studies seem to suggest that CHr is an accurate method of assessing IDE in pregnancy, the effects of suboptimal folate or vitamin B12 status may be important sources of error during pregnancy that require further study.

CHr is essentially a short-term indicator, but it can now be measured alongside the percentage of hypochromic erythrocytes, which until recently had only been available by manual blood film (92). Together CHr and the percentage of hypochromic erythrocytes appear to offer the most accurate assessment of past and present IDE and IDA during pregnancy. However, determination of the underlying cause of IDE may require additional assessment of tissue Fe levels.

**Assessment of maternal tissue iron depletion**

The maintenance of serum Fe concentrations is dependent on the export of Fe from storage cells such as the duodenal mucosa, macrophages and hepatocytes (93). As these stores become depleted, the proportion of serum transferrin saturated with Fe (transferrin saturation) decreases. In response, the serum concentration of transferrin (the total Fe-binding capacity) is increased to promote uptake and delivery of Fe to dependent tissues such as the bone marrow or placenta. The classical signs of tissue Fe deficiency are therefore low serum Fe (<400 μg/l), high serum total Fe-binding capacity (>2160 μg/l), and low transferrin saturation (<16%) (37,94).

By mid–late pregnancy tissue Fe deficiency is common as a result of sustained demand for Fe during the expansion
of the erythrocyte mass and development of the conceptus. However, during the last trimester (27–40 weeks) the fetus starts to grow at its maximal rate and accumulates Fe stores in preparation for infancy(49,57). If during this period maternal serum Fe concentrations are suboptimal, placental Fe uptake may be maintained by up-regulation of placental transferrin receptors(95,96) and modulation of Fe regulation in the maternal gut(97). However, such mechanisms are not always able to compensate sufficiently to prevent reduced rates of fetal Fe accretion(98,99). Accurate identification of maternal tissue Fe deficiency during pregnancy is therefore essential to prevent suboptimal Fe status in the infant.

Serum total Fe-binding capacity is increased by steroid hormones such as oestrogens(99), which during pregnancy are found in high concentrations in maternal plasma. Even Fe-replete women with constant serum Fe tend to show increasing total Fe-binding capacity and falling transferrin saturation with advancing gestation(98,100–102). As a result it may be preferable during pregnancy to concentrate entirely on the direct measurement of serum Fe(103). Serum Fe measures the transferrin-bound Fe available for maternal and placental uptake. It has a high level of intra-individual and diurnal variability, and may reflect recent dietary intake, especially following use of supplemental Fe(104,105). It is highly reactive to infection and inflammation since both Fe uptake from the gut and Fe release from macrophages are down-regulated by the action of hepcidin, a regulator of Fe homeostasis(106). These limitations may be overcome to some extent by sufficient sample size, careful study design and repeated measurements. However, its susceptibility to such large day-to-day fluctuations makes it a crude indicator of maternal tissue Fe status and of little diagnostic utility in the individual.

A general summary of Fe biomarkers during pregnancy is shown in Table 1.

### Table 1. Laboratory measures of maternal iron status

<table>
<thead>
<tr>
<th>Body Fe storage</th>
<th>Tissue Fe depletion</th>
<th>Fe-deficient erythropoiesis</th>
<th>Total body Fe</th>
</tr>
</thead>
<tbody>
<tr>
<td>‘Adequacy’ should be based on</td>
<td>[↑sTfR &gt; 8.5 mg/l]</td>
<td>Short-term indicators used for</td>
<td>Calculated according to Skikne</td>
</tr>
<tr>
<td>current Fe stores as measured</td>
<td>[↓Total body Fe &lt; 0 mg/kg]</td>
<td>assessment of impaired Hb</td>
<td>et al.(108); body Fe (mg/kg) =</td>
</tr>
<tr>
<td>by serum ferritin, relative to</td>
<td>[↓Serum Fe &lt; 400 μg/l]</td>
<td>synthesis:</td>
<td>7.28229)/0.1207.</td>
</tr>
<tr>
<td>future needs during pregnancy</td>
<td>[↑Serum ferritin &lt; 12 μg/l]</td>
<td>TZnPP ≥ 60 μmol/mol haem</td>
<td>Possible error during</td>
</tr>
<tr>
<td>1st Trimester &gt; 75 μg/l</td>
<td>no longer linear but</td>
<td>JCHR ≤ 28.8 pg</td>
<td>pregnancy as a result of</td>
</tr>
<tr>
<td>3rd Trimester &gt; 30 μg/l</td>
<td>still associated with</td>
<td>Long-term indicators used for</td>
<td>erythropoiesis and</td>
</tr>
<tr>
<td>Markers of inflammatory status</td>
<td>tissue deficiency</td>
<td>assessment of anaemia:</td>
<td>inflammation.</td>
</tr>
<tr>
<td>useful to establish reliability</td>
<td>sTfR not significantly</td>
<td>[JMVC &lt; 80 fl]</td>
<td>Useful indicator for intervention</td>
</tr>
<tr>
<td>of values; bias likely during</td>
<td>affected by inflammation,</td>
<td>[JHb &lt; 110 g/l; &lt; 105 g/l]</td>
<td>studies to improve maternal</td>
</tr>
<tr>
<td>late pregnancy</td>
<td>unlike other indicators.</td>
<td>(2nd trimester)</td>
<td>Fe status.</td>
</tr>
<tr>
<td></td>
<td>sTfR:ferritin</td>
<td>JHct &lt; 33.0%; &lt; 32.0%;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>will incorporate acute</td>
<td>(2nd trimester)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>phase reactivity of ferritin</td>
<td>↓HYPO% &gt; 3.4%</td>
<td></td>
</tr>
</tbody>
</table>

sTfR, soluble transferrin receptor; ZnPP zinc protoporphyrin; CHr, reticulocyte Hb content; MCV, mean corpuscular volume; Hct, packed cell volume; percentage saturation with advancing gestation(80,100–102). As a result it may be preferable during pregnancy to concentrate entirely on the direct measurement of serum Fe(103).

Serum sTfR has a high specificity to tissue Fe deficiency and a low sensitivity to acute or chronic inflammation, unlike most other indicators of Fe status(110,111). When combined with measurement of serum ferritin, the sTfR:ferritin can be used to estimate total body Fe (mg/kg) with high precision(112).

However, the accuracy of sTfR and sTfR:ferritin during pregnancy has not yet been firmly established. As a large proportion of sTfR is derived from erythroid precursors in the bone marrow, changes in the rate of erythropoiesis also affect sTfR concentrations. In Fe-replete individuals sTfR is an accurate indicator of erythropoiesis(113), whereas in those individuals with a steady rate of erythropoiesis sTfR is an accurate indicator of tissue Fe depletion(114).

Unfortunately, pregnancy is associated with marked changes in both erythropoiesis and body Fe stores that introduce considerable uncertainty as to the primary cause of changes in sTfR concentrations. There are few data on the normal patterns of erythropoiesis during pregnancy, mainly because of the prohibitive nature of radio Fe studies, and therefore it is not possible to adjust for the influence of erythropoiesis at each stage. During pregnancy raised sTfR may in fact be more strongly predicted by the rate of erythropoiesis than by Fe status(115).

Some studies have reported sTfR to be a sensitive and specific measure of tissue Fe deficiency in pregnancy(116,117), but these have not had the benefit of comparison with gold standard methods, as has been the case for NPNL subjects. sTfR and the sTfR:ferritin appear to have great potential as indicators of tissue Fe depletion, but their validity during pregnancy remains uncertain until the effects of erythropoiesis can be ascertained. In the meantime, sTfR:ferritin is probably of most use as a method of
evaluating the effectiveness of interventions to improve Fe status during pregnancy, since the error as a result of erythropoiesis and inflammation ought to be similar in both control and intervention groups\(^{(12)}\). Although folate-binding protein assays have been widely used, persistent doubts have been raised about their reliability\(^{(128–130)}\). A round-robin international survey of reputable laboratories found inter-assay CV of 32–41\% with RCF and 17–48\% with serum folate\(^{(131)}\). The variability of high or low values was found to be much greater than that for mid-range concentrations. Much of the difference has been attributed to the types of reference standards in commercial assays, which were found to vary considerably by manufacturer. Recent efforts to standardise these reference materials in whole blood\(^{(122)}\) and serum\(^{(133)}\) may substantially reduce inter-assay variability in the future, thus making comparison of absolute values between studies more reliable.

**Direct assessment of folate status**

Most pregnant women in the UK will only be screened for folate deficiency by assessment of MCV. However, such assessment is highly insensitive, especially in the presence of concurrent Fe deficiency, and is unable to specify the underlying cause. The most common method of directly assessing folate status is serum or plasma folate. This short-term indicator is highly sensitive to recent folate intake and indicates the amount of folate being taken up by tissues, catabolised and excreted at the point in time when the serum sample was drawn\(^{(118)}\). It is markedly and quickly decreased during negative folate balance, when tissue demands exceed that provided by dietary supply\(^{(119)}\).

The advantage of serum folate in studies of pregnant women is that it represents the concentration available for placental uptake at the time of blood sampling, whereas erythrocyte folate (RCF) represents average availability over several months. As folate demands change several fold between trimesters, analysis of serum folate allows time-specific determination of folate availability. In NPNL individuals serum folate and dietary folate intake are generally well correlated. However, during pregnancy this correlation may be weakened\(^{(120)}\). RCF concentrations indicate the availability of folate at the time when the cell was forming in the bone marrow\(^{(123)}\). Once DNA synthesis ceases in maturing erythrocytes, the remaining folate concentrations are relatively static until erythropoiesis, when the folate is recycled. The mean RCF concentration therefore reflects a moving average of serum folate status over the 120d life-span of the erythrocyte population. As this period also approximately corresponds to the time in which hepatic folate stores become depleted on a folate-free diet\(^{(122)}\), RCF concentrations are assumed to correlate with those in tissue\(^{(123)}\); this correlation has been confirmed by examination of liver and RCF concentrations in subjects with chronic alcoholism\(^{(124)}\). However, the validity of this assumption during late pregnancy, when both folate and erythrocyte turnover are markedly different from that during the NPNL state\(^{(125)}\), remains untested.

RCF is standardised by packed cell volume and is therefore not confounded by variation in plasma volume expansion. Values \(<140–150\mu g/l\) for RCF or \(<3\mu g/l\) for serum folate are generally considered to be indicative of folate deficiency in the NPNL state\(^{(126)}\). Both serum folate and RCF are most often measured by methods that use high-affinity folate-binding proteins such as RIA, ion-capture assays and cheluminescence. Microbiological assays using various strains of Lactobacillus casei have traditionally been used as the reference standard and some are now fully automated and cost efficient\(^{(127)}\). However, they can sometimes be confounded by antibiotics, the use of which may be relatively common during pregnancy, and are therefore not often used in clinical contexts.

Although folate-binding protein assays tend to underestimate folate status as a result of incomplete recovery of 5-methyltetrahydrofolate\(^{(134)}\), they may actually overestimate folate status during pregnancy. A comparison of four different methods for assessing RCF in pregnant and non-pregnant women\(^{(135)}\) has reported major inter-assay differences, with RIA giving approximately 40\% greater values than L. casei\(^{(136)}\). Whilst the folate status of pregnant and non-pregnant women were not found to be significantly different when measured using L. casei and GC–MS, the values for pregnant women according to the folate-binding-protein assays were found to be higher than those of the non-pregnant women.

A recently-developed method using isotope-dilution liquid chromatography–tandem MS now enables high-throughput analysis of folate status with high precision and the ability to differentiate between folate species\(^{(136)}\). This method represents a considerable advance in the ability to examine the complex inter-relationships between folate species and may help to resolve the emerging debate about the risks associated with unmetabolised folic acid in plasma\(^{(137,138)}\). Recent comparative studies have shown excellent agreement between liquid chromatography–MS–MS and L. casei\(^{(134)}\).

The lack of comparability between laboratories has led to the classification of inadequate folate status being generally based on comparison with reference data from local populations and not on symptoms of functional folate deficiency such as neutrophil hypersegmentation or macrocytosis. Such an approach may be preferable since during pregnancy these functional indices are less reliable than during the NPNL state. Neutrophils tend to hypo-segment with advancing gestation, which can mask folate deficiency\(^{(139)}\), and MCV may sometimes be more influenced by the microcytic effects of poor maternal Fe status during late pregnancy, as seen in pregnant adolescents from the About Teenage Eating Study (Fig. 1). However, the use of reference population data to determine folate status is complicated by the increasing amount of food now fortified with folic acid in modern diets, especially fortified flour, since normal ranges in some populations are now higher than those of previous generations\(^{(140)}\). As a result, the use of normality-based approaches is not only incompatible between countries with different fortification strategies, but is unlikely to be a sensitive method of detecting functional folate deficiency in pregnancy.
can reflect recent intake, especially when consumed in relatively high doses such as those found in antenatal folic acid supplements (146).

During pregnancy the correlation between folate status and tHcy becomes weaker with advancing gestation. Serum tHcy falls markedly during the first 20 weeks of gestation to approximately half that of NPNL levels and then increases slowly back to pre-conceptual levels by parturition (147). This uncoupling with folate concentrations, which in unsupplemented subjects continue to fall throughout pregnancy, remains poorly understood. The decline in serum tHcy in the first half of pregnancy has been suggested to be the result of plasma volume expansion, increased renal clearance, decreased serum albumin and higher concentrations of oestrogens, most of which have been eliminated as causal factors (173). Oestrogens have been suggested as the most probable mediators, but their homocysteine-lowering effects have recently been questioned (146,149).

Despite its idiosyncratic behaviour during pregnancy, serum tHcy remains a sensitive functional biomarker of folate status in pregnant women. Precision may be further increased by adjustment for confounding factors such as renal function, smoking, vitamin B₁₂ status and coffee intake (150,151). A plasma tHcy concentration >15 μmol/l is generally used to define mild hyperhomocysteinaemia in NPNL women, but because of the physiological decrease in plasma tHcy concentrations during pregnancy, an upper reference limit of 10 μmol/l has been recommended by an expert panel (150). Whilst there have been problems with tHcy assessment in the past, most modern assays now have good levels of precision and comparability (150).

**p-Amino- and acetamido-benzoylglutamate**

The rate of folate turnover can be quantified by measurement of the folate catabolites p-amino-benzoylglutamate and acetamido-benzoylglutamate present in serum or urine (152). Until recently, the analysis procedure has required extensive sample preparation and clean up (153), making it unsuitable for clinical or epidemiological use. However, recent innovations using liquid chromatography–MS–MS have simplified this process greatly, such that high-volume analysis of urinary and serum p-amino-benzoylglutamate and acetamido-benzoylglutamate may be both possible and cost effective in the future (154,155).

Analysis of p-amino-benzoylglutamate may also have potential as a biomarker of folate status in degraded blood samples (155). Measurement of folate turnover is not a measurement of folate status per se. It does, however, provide detailed information on an individual’s folate requirement, since the amount excreted can be assumed to be the amount required to replace it in order to prevent depletion. The analysis of p-amino-benzoylglutamate and acetamido-benzoylglutamate, together with conventional assessment of folate status, may enable more detailed examination of the relationships between folate demands over the course of pregnancy and maternal folate status, which hitherto has been restricted to samples of less than ten individuals (152,156).
**Poor folate status and pregnancy outcomes**

The placenta is rich in high-affinity folate receptors that are able to maintain folate delivery to the fetus even at relatively low maternal plasma concentrations\(^{(142)}\). However, cord and placental folate concentrations are still dependent on those in maternal plasma\(^{(157)}\). Poor folate status has been strongly associated with several adverse pregnancy outcomes, such as preterm delivery, pre-eclampsia, stillbirth, spontaneous abortions, placental abruption, fetal growth restriction and congenital birth defects\(^{(158}-162\)). Raised tHcy is also associated with these risks, although it is not yet clear what proportions are the result of poor folate status per se or to the folate-independent actions of homocysteine\(^{(156,157)}\). The preventive effect of folic acid supplementation in substantially reducing the risk of neural-tube defects has been proven in several randomised controlled trials\(^{(163,164)}\). However, whilst the reduction in the incidence of neural-tube defects has been a tremendous accomplishment of nutrition research, it has also resulted in the situation in which it is now unethical to conduct placebo-controlled intervention trials to determine the effects of folic acid supplementation during pregnancy, since the periconceptual use of 400 µg folic acid is now part of routine antenatal care. Hence, the ability to test the associations between poor folate status and other pregnancy outcomes under controlled conditions is severely limited.

**Assessment of vitamin B_12_ status**

**Serum cobalamin**

The accurate detection of vitamin B\(_{12}\) deficiency during pregnancy is extremely difficult. In normal NPNL subjects and in patients with pernicious anaemia serum cobalamin generally correlates well with tissue concentrations, although kinetic studies have shown that concentrations in serum tend to be preserved at the expense of those in tissue\(^{(165,166)}\). As a result, low serum cobalamin is only likely to become apparent with moderate or severe tissue deficiency. Serum cobalamin of <150 ng/l is generally associated with clinically-apparent deficiency in the NPNL state, yet the usefulness of this, or indeed any, cut-off during pregnancy is questionable\(^{(167)}\). Concentrations below this level have been found in pregnant women with dietary intakes well in excess of the US RDA of 2.6 µg/d and no corroborative clinical or biochemical evidence of deficiency\(^{(168,169)}\).

**Functional biomarkers: serum total homocysteine and serum methylmalonic acid**

As serum cobalamin is less sensitive during pregnancy, mild or occult vitamin B\(_{12}\) deficiency may be more easily detected with functional biomarkers such as serum methylmalonic acid and serum tHcy. Vitamin B\(_{12}\) has only two functions in animal cells and impairment of either causes the accumulation of unmetabolised precursors. Methylmalonic acid becomes elevated as a result of the impairment of the cobalamin-dependent enzyme methylmalonyl-CoA mutase, which converts methylmalonyl-CoA to succinyl-CoA, an essential precursor of haem and an intermediate in the citric acid cycle. Serum tHcy accumulates as a result of the impairment of cobalamin-dependent methionine synthase\(^{(170)}\). Although methylmalonic acid and tHcy are more sensitive than serum total cobalamins, both have major limitations during pregnancy. Serum tHcy can be elevated by folate deficiency, which is more common than vitamin B\(_{12}\) deficiency in Western countries, and becomes uncoupled from vitamin B\(_{12}\) status with advancing gestation. Methylmalonic acid is also affected by renal clearance as well as concurrent use of antibiotics\(^{(171)}\), and can sometimes be sporadically elevated during pregnancy for reasons as yet unknown\(^{(167,168)}\). Measurement of methylmalonic acid is also expensive and requires the use of techniques that may not be widely available in clinical laboratories. The use of either biomarker alone is therefore insufficient to determine vitamin B\(_{12}\) status during pregnancy, although they can provide useful supplemental data to other more direct measures.

**Holo-transcobalamin**

Cobalmins in human serum are transported by the two main binding proteins, haptocorrin and transcobalamin (TC). These proteins are either complexed with cobalmins (holo) or cobalamin-free (apo). Haptocorrin carries most of the cobalmins in serum, which are cleared slowly over a period of several days. However, no major function has yet been attributed to it other than the binding of metabolically-inert forms of vitamin B\(_{12}\). TC, on the other hand, is highly active and serves as the main transporter of cobalmins from the intestine to tissues including the placenta, which is rich in TC receptors\(^{(172)}\). Newly-absorbed cobalmins bound to holo-TC are generally cleared from the maternal circulation within minutes, although a small amount remains in circulation.

Recent longitudinal analysis of TC and haptocorrin fractions during pregnancy has determined the reason for the decline in serum cobalamin with advancing gestation\(^{(7)}\). Large reductions were found in the haptocorrin fraction saturated with true cobalmins, whereas holo-TC remained stable over the whole of gestation. This finding explains why the physiological reduction in serum cobalamin during pregnancy is not associated with functional vitamin B\(_{12}\) deficiency, as haptocorrin is relatively inert.

Since TC is the main fraction available for placental uptake of vitamin B\(_{12}\), maternal serum holo-TC concentrations are highly correlated with cord blood cobalmins and therefore reflect the maternal supply of vitamin B\(_{12}\) to the fetus\(^{(172)}\). Whilst the high sensitivity and specificity of maternal holo-TC makes it the most suitable biomarker for the assessment of maternal vitamin B\(_{12}\) status, the assay required for its assessment is still highly-specialised and not yet widely available. Until such time as it is suitable for high-volume low-cost throughput of samples, routine screening for maternal vitamin B\(_{12}\) deficiency will continue to be carried out using crude indicators with low sensitivity and specificity such as MCV and serum cobalamin, leaving many pregnant women at risk of subclinical vitamin B\(_{12}\) deficiency.
Implications of poor vitamin B₁₂ for fetal growth

With the notable exception of vegans, low vitamin B₁₂ intakes are relatively rare in Western countries and consequently poor vitamin B₁₂ status is normally a result of non-dietary factors. However, poor vitamin B₁₂ status is still a major public health problem in developing countries with low consumption of animal foods, such as India and Nepal. Studies in these countries have found similar relationships between vitamin B₁₂ status and fetal growth restriction to those found with folate, although its relationships with other pregnancy outcomes remain unknown.

Summary

Interpretation of micronutrient biomarkers and indices during pregnancy is complex and should take into account the maternal, placental and fetal adaptations to pregnancy, most of which are highly variable between individuals and dependent on the gestational age at which measurement occurs. As a result of the complexity and uncertainty surrounding these factors, sensitivity and specificity of virtually all biomarkers will be reduced, especially during late pregnancy, and many of the normal ranges commonly used for NPNL individuals may be inappropriate. Current antenatal screening of micronutrient status in the UK is crude, as it is concerned primarily with the treatment rather than prevention of nutritional anaemia.

Future research should establish methods that can identify recent-onset functional deficiency without substantial bias from maternal inflammation, infection, endocrine influences, plasma volume and renal function. Several relatively new indicators of Fe status show much promise in this context, especially when used in combination. However, they require further validation in pregnant subjects. Assessment of folate status remains problematic because of issues surrounding the reliability and comparability of folate-binding-protein assays. However, new methods using liquid chromatography–MS–MS are able to measure specific folate species, including products of folate catabolism, with high precision and in large sample sizes. Similarly, measurement of serum holo-TC may provide much needed data on the relationships between vitamin B₁₂ status and pregnancy outcome.

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