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The erythrocyte incorporation of absorbed non-haem iron in pregnant women

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Studies of Fe absorption in pregnancy often make unfounded assumptions of erythrocyte incorporation. Therefore, we measured the absorption and utilisation of Fe during early and late pregnancy by the erythrocyte incorporation of two stable isotopes. 8·5 mg ^{57}Fe (oral) and 0·5 mg ^{58}Fe (intravenous) were given to five non-pregnant women, to five women in early gestation (12 weeks) and five women in late gestation (36 weeks). The stable isotope ratios in whole blood 14 d later were measured by MS. Together with estimation of body Fe mass, this enabled the calculation of Fe absorption and erythrocyte incorporation. In non-pregnant women, Fe absorption averaged 20·3 (range 10·2–34·3) %. It was not significantly different in early pregnancy (11·8 (range, 4·4–24·8) %), but during late pregnancy Fe absorption increased to 59·0 (range 38·2–77·2) %. All non-pregnant and early-pregnancy subjects had normal Fe status, but two women in late pregnancy had evidence of Fe insufficiency. During early and late pregnancy, mean erythrocyte incorporation was 63·4 (SD 12·1) % and 71·0 (SD 10·4) % respectively, significantly reduced ($P = 0·003$) compared with non-pregnant subjects (90·1 (SD 6·0) %). Decreased erythrocyte incorporation of absorbed Fe in early pregnancy is compatible with reduced Fe demand and low oral absorption. However, during late pregnancy decreased erythrocyte incorporation associated with high absorption and Fe insufficiency is different from the high erythrocyte incorporation which occurs in non-pregnant Fe-deficient women. This suggests that part of the aetiology of Fe deficiency during pregnancy may be the reduction of Fe utilisation.

Iron absorption: Pregnancy: Erythrocyte: Stable isotopes: Mass spectrometry

Studies of Fe absorption in pregnancy have often made assumptions of erythrocyte incorporation based on non-pregnant data. The purpose of the current study was to document the changes in erythrocyte incorporation and assess the implications for the aetiology of pregnancy anaemia, since the increase in Fe absorption in normal pregnancy may not always prevent Fe deficiency (Barrett *et al.* 1994).

The incorporation of isotopes of Fe into the haemoglobin (Hb) of erythrocytes is a well-established method of measuring Fe absorption (Hosain *et al.* 1967). Oral administration of a single isotope and subsequent enrichment of isotope ratios in erythrocytes measured 2 weeks later allows semi-quantitative estimates of Fe absorption, of use mainly in comparative studies of Fe absorption and availability from different foods. One must assume how much of a given isotope is incorporated into erythrocytes, an incorporation which may alter from between 74 and 94 % (Lunn *et al.* 1967) in non-pregnant individuals to 65 % (Dyer & Brill, 1972) in subjects during late pregnancy. More accurate measurements require the intravenous (iv) administration of a second isotope to account for the redistribution of the orally absorbed Fe between erythrocytes and the rest of the body and have been validated by comparison with whole-body counting using radio-isotopes (Lunn *et al.* 1967; Larsen & Milman, 1975; Heinrich & Fischer, 1982).

Most stable isotope studies have used the single isotope ^{58}Fe administered orally, and absorption has been calculated from the change in the ^{58}Fe:^{57}Fe ratio in erythrocytes using inductively coupled plasma MS (Fomon *et al.* 1988) or neutron activation analysis (Dyer & Brill, 1972; Fairweather-Tait & Minski, 1986). The use of oral ^{54}Fe and fast atom bombardment MS (Lehmann *et al.* 1988) and two oral

Abbreviations: Hb, haemoglobin; iv, intravenous; PCV, packed cell volume.

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isotopes ^{57}Fe and ^{58}Fe using thermal ionisation MS has been reported (Kastenmayer *et al.* 1994). We have shown that administration of physiological doses of ^{57}Fe orally and ^{58}Fe iv gave reliable enrichment in erythrocytes measured by inductively coupled plasma MS (Whittaker *et al.* 1992) and we have applied this method to Fe absorption studies (Barrett *et al.* 1992). This has been followed by a dual isotope study of Fe supplementation in late pregnancy using thermal ionisation MS (O'Brien *et al.* 1999). We now report the clinical application of this method to both pregnant and non-pregnant subjects with due consideration of Fe incorporation at physiological doses.

Materials and methods

Subjects

Fifteen healthy women between the ages of 22 and 34 years volunteered for the study. Five were non-pregnant, five were between 10 and 12 weeks pregnant and five were between 36 and 37 weeks pregnant. Gestational age was calculated from the first day of the last menstrual period and confirmed by sonar measurement of fetal size. No woman had been pregnant before and no subject had a history of any medical illnesses, or if pregnant, of any obstetrical complication. None of the non-pregnant subjects were taking the oral contraceptive pill and no subject was taking vitamin or Fe supplementation. All were non-smokers. All recruits gave informed consent and the project had ethical approval from the Combined Ethics Committee of the Newcastle Regional Health Authority and Newcastle University.

Study protocol

For 3 d before and 2 d during the study each subject followed a diet plan allowing the ingestion of not more than 5 mg Fe/d. This was done in order to reduce variation in Fe intake between individuals prior to testing and to optimise isotope absorption by making the enriched isotope not an undue addition to the normal dietary intake. Following an overnight fast, the subjects attended the research unit and were seated comfortably in a warm room for 15 min before an iv cannula was inserted in each arm. Blood (10 ml) was aspirated and placed in a lithium heparin tube to serve as the sample from which the basal isotope ratios would be measured. Blood was also taken sufficient to allow measurement of the woman's full blood count, serum ferritin and erythrocyte Zn protoporphyrin using established methods (Whittaker *et al.* 1996), as well as to provide serum to serve as a blank sample for use in the determination of the subject's plasma volume. The plasma volume was then measured using Evan's Blue dilution (Whittaker & Lind, 1993) 10 min after injection. Erythrocyte mass (derived from plasma volume and packed cell volume (PCV)), blood volume, Hb mass and total Fe mass were calculated (Paintin, 1963; Whittaker *et al.*1996):

$$\text{blood volume (ml)} = \text{plasma volume (ml)} \times (100/(100 - (\text{PCV (\%)} \times 0{\cdot}88))),$$

$$\text{total Fe mass (mg)} = \text{blood volume (l)} \times \text{Hb (g/l)} \times 3{\cdot}47 \text{ (Fe in Hb (mg/g)}.$$

The isotopes ^{57}Fe for oral use and ^{58}Fe for iv use were prepared for administration as aqueous ferrous sulfate with added ascorbic acid (3 g/l) as described previously (Barrett *et al.* 1992). Isotopic composition of the tracers was for enriched ^{57}Fe (%):^{54}Fe 0, ^{56}Fe 0·57, ^{57}Fe 95·93, ^{58}Fe 3·5; for enriched ^{58}Fe (%):^{54}Fe 0, ^{56}Fe 0·21, ^{57}Fe 6·56, ^{58}Fe 93·23.

An ampoule of ^{58}Fe (256 μg in 2 ml with 6 mg ascorbic acid, pH 1·7) was taken up into a syringe with 10 ml normal saline and given by iv injection. After 5 min a 5 ml ampoule containing 4·7 mg ^{57}Fe and 15 mg ascorbic acid was emptied into a glass containing 50 ml fresh orange juice (25 mg ascorbic acid) which was drunk by the subject. This procedure is standard for administration of reference doses of Fe (Cook *et al.* 1991). The ampoule and glass were weighed before and after use to give the amount of isotope ingested. No food, tea or coffee were allowed for 2 h. The following morning, again after an overnight fast, and following the same diet plan, the two isotopes were given in a similar fashion. The oral dose of ^{57}Fe was spread over 2 d, 5 mg being equivalent to a typical meal, and other Fe intake was reduced to 5 mg/d so that total intake remained undisturbed. Following the second test dose of Fe, subjects reverted to their normal diet. After 14 d, 10 ml blood was taken from each subject, and the enriched Fe isotope ratios were measured. By 14 d, studies have shown (Hosain *et al.* 1967) that tracer, absorbed and incorporated into erythrocytes, is at a stable peak.

Sample preparation and analysis

Aqueous solutions of whole blood (1:25, v/v) were prepared according to our published method (Whittaker *et al.* 1992). They were analysed using the inductively coupled plasma MS instrument PlasmaQuad PQ2+ (VG Elemental, Winsford, Ches., UK). Details of the operating conditions have been described and include a mass range of 51–56 Da and an analysis time of 2 min/sample replicate, ten replicates in all. In order to obtain Fe isotope ratios with a precision of $<0{\cdot}5$ %, the inductively coupled plasma MS instrument had to be optimised such that $^{40}Ar^{16}O$ was reduced to a minimum and the Fe concentration in the samples had to be sufficiently high to produce enough signal to be measured with the required precision without saturating the detection system. A preparation of natural Fe (atomic absorption standard; Sigma, Poole, Dorset, UK) at a concentration of 10 mg/l was used to calculate the Fe concentration in the samples. The mean isotope ratios of this standard were used for any bias correction of isotope ratios in the samples. The standard has no certified isotopic composition, but deviation from the internationally accepted representative isotopic composition (Commission on Atomic Weights and Isotopic Abundances, 1997), in

practice an average value of 4 % for ^{57}Fe:^{56}Fe and 15 % for ^{58}Fe:^{57}Fe was used to adjust the isotope ratios of the unknown samples. Since this study was completed, a certified isotopic reference material for Fe has become available (IRM-014; EU Institute of Reference Materials, Geel, Belgium). Limits of detection (Miller & Milller, 1993) were set at 3·3 SD above basal ratios for each sample, to ensure (with at least 95 % CI) that changes in isotope ratios in 2-week samples were a result of isotope enrichment and not due to measurement imprecision.

Calculation of Fe absorption

The absorption of the oral dose of Fe was calculated by measuring the change in the ^{57}Fe:^{56}Fe ratio over the 2-week period as well as the basal Fe mass. This was then adjusted for the redistribution of absorbed isotope by the amount of ^{58}Fe recovered 2 weeks after the iv injection and for the small amounts of ^{57}Fe present (6·6 %) in the iv preparation and of ^{58}Fe present (3·5 %) in the oral preparation. The derivation of this formula has been previously explained (Barrett *et al.* 1992):

$$\text{total oral absorption of } {}^{57}\text{Fe} = \left(\frac{\text{(change in } {}^{57}\text{Fe:}{}^{56}\text{ Fe)}}{\text{(change in } {}^{58}\text{Fe:}{}^{56}\text{ Fe)}} \times {}^{58}\text{ Fe given (iv + oral))} - {}^{57}\text{ Fe given (iv)},$$

$$\text{and incorporation of } {}^{58}\text{Fe} = \frac{\text{(total circulating } {}^{56}\text{Fe mass} \times \text{change in } {}^{58}\text{Fe:}{}^{56}\text{ Fe)}}{\text{Fe given (iv)}}.$$

Statistics

Results are given as mean values and standard deviations or geometric mean values (range) for Fe absorption and serum ferritin. Statistical comparison of groups was by one-way ANOVA with multiple comparisons controlled using the Student-Newman-Keuls method (SigmaStat for Windows; SPSS Science, Chicago, IL, USA). Associations were assessed by linear regression. The significance level (two-sided) was $P < 0{\cdot}05$.

Results

Isotope ratios, measurement precision and results of Fe absorption and incorporation are shown in Table 1. The mean basal ^{57}Fe:^{56}Fe and ^{58}Fe:^{56}Fe ratios and their measurement precision were not significantly different between groups (Table 1). The average precision of the ^{57}Fe:^{56}Fe ratios (0·25 %) was equivalent to a minimal detectable absorption of 3 %. Clinical details and indices of Fe status are given in Table 2.

Non-pregnant subjects

The measured absorption ranged from 10 to 34 % (geometric mean value 20 %). Using the Fe mass derived from the dye-dilution technique the percentage of the iv dose that was incorporated into the erythrocytes ranged from 83 to 97 % (mean value 90 %). All subjects had Hb and PCV concentrations within normal limits (Centers for Disease Control, 1989). The subject with the highest ferritin (89 μg/l) had the lowest absorption (10·2 %), while the subject with a low ferritin concentration suggestive of storage Fe depletion (7 ug/l) had an absorption of 24·9 %, above the mean value.

Pregnant subjects

The mean increase in the ^{58}Fe:^{56}Fe ratio in response to the iv doses did not differ between early and late pregnancy

Table 1. Erythrocyte iron isotopic enrichment and iron absorption during pregnancy*
(Mean values and standard deviations for five subjects with ten replicate measurements on each occasion)

	Non-pregnant		12 weeks gestation		36 weeks gestation	
	Mean	SD	Mean	SD	Mean	SD
Basal ^{57}Fe:^{56}Fe	0·02395	0·00009	0·02385	0·00017	0·02386	0·00006
SD (within subjects)	0·00005		0·00007		0·00005	
Precision ^{57}Fe:^{56}Fe (%)	0·22	0·07	0·28	0·14	0·19	0·06
Detection limit (3·3 SD)	0·02411		0·02405		0·02400	
^{57}Fe:^{56}Fe at 2 weeks	0·02511	0·00057	0·02443	0·00043	0·02594	0·00069
SD (within subjects)	0·00005		0·00008		0·00007	
Precision ^{57}Fe:^{56}Fe (%)	0·20	0·08	0·33	0·12	0·28	0·09
Ratio increase	0·00116	0·00052	0·00058	0·00041	0·00208	0·00069
Increase (SD multiple)	23·4		9·1		46·1	
Basal ^{58}Fe:^{56}Fe	0·00295	0·00005	0·00301	0·00001	0·00302	0·00002
SD (within subjects)	0·00001		0·00001		0·00001	
Precision ^{58}Fe:^{56}Fe (%)	0·47	0·12	0·45	0·21	0·34	0·07
Detection limit (3·3 SD)	0·00299		0·00305		0·00305	
^{58}Fe:^{56}Fe at 2 weeks	0·00330	0·00006	0·00328	0·00004	0·00330	0·00005
SD (within subjects)	0·00002		0·00001		0·00001	
Precision ^{58}Fe:^{56}Fe (%)	0·48	0·16	0·42	0·17	0·34	0·20
Ratio increase	0·00035	0·00004	0·00027	0·00004	0·00028	0·00006
Increase (SD multiple)	27·2		25·3		27·8	

* For details of subjects and procedures, see p. 324. Enrichments were measured 2 weeks after isotope administration.

Table 2. Oral iron absorption and iron status during pregnancy*
(Mean values and standard deviations for five subjects)

	Non-pregnant		12 weeks gestation		36 weeks gestation	
	Mean	SD	Mean	SD	Mean	SD
Height (m)	1·63	0·04	1·66	0·03	1·64	0·06
Weight (kg)	57·4	2·8	63·2	11·4	74·3*	9·4
Hb (g/l)	138	6	123††	6	120††	12
Packed cell volume (l/l)	0·404	0·021	0·358††	0·019	0·357††	0·027
Ferritin (μg/l)	33·8	7·0–89·0†	26·7	10·0–86·0†	12·3	5·0–89·0†
Mean cell volume (fl)	87·9	4·5	89·8	2·9	87·2	3·4
ZPP (μg/g Hb)	1·9	0·2	1·6	0·4	2·6**	0·6
Blood volume (ml/kg)	64·4	3·4	64·4	8·4	72·9	5·6
Hb mass (g)	508	43	494	45	650**	107
Oral dose ^{57}Fe (mg)	8·48	0·41	8·23	0·27	8·90	0·09
Dose ^{58}Fe iv (mg)	0·52	0·01	0·52	0·01	0·51	0·01
^{57}Fe oral absorption (%)	20·3	10·2–34·3†	11·8	4·4–24·8†	59·0**	38·2–77·2†
^{58}Fe iv recovery (%)	90·1	6·0	63·4††	12·4	71·0††	10·6

Hb, haemoglobin, ZPP, zinc protoporphyrin; iv, intravenous.
* For details of subjects and procedures, see p. 324.
† Geometric mean value (range).
Mean values were significantly different from those in preceding columns (ANOVA): ** $P < 0{\cdot}01$.
Mean values were significantly different from those of the non-pregnant group (ANOVA): †† $P < 0{\cdot}01$.

and the non-pregnant subjects. The mean increase in the ^{57}Fe:^{56}Fe ratio and the calculated oral Fe absorption was significantly higher ($P = 0{\cdot}002$) in the subjects in late pregnancy (range 38–77 %) compared with those in early pregnancy (range 4–25 %) and to those who were not pregnant. At 12 weeks gestation, the lower enrichment of the erythrocyte ^{57}Fe:^{56}Fe ratio and the calculated absorption did not reach statistical significance when compared with the non-pregnant subjects. The percentage of the iv dose that was incorporated into the erythrocytes at 12 and 36 weeks gestation ranged from 47 to 78 % and 62 to 88 % respectively, both being significantly lower ($P = 0{\cdot}003$) than the recovery measured in the non-pregnant subjects.

The average blood volume (ml) and Hb mass were not different between non-pregnant subjects and those subjects studied during early pregnancy, but in the subjects studied during late pregnancy total blood volume was greater on average by 35 % (3 995 (SD 211) *v.* 5 384 (SD 518) ml, $P < 0{\cdot}001$) and Hb mass (g) by 32 % ($P = 0{\cdot}008$, Table 2). Body weight was 18 % greater in the late pregnancy compared with the early pregnancy group, proportionately less than the rise in Hb mass. Individual measurements of blood volume expressed per unit weight varied from 54 to 81 ml/kg, but the wide variation meant that comparison of the three group means was not significant ($P = 0{\cdot}075$). Examination of individual results showed that a reduction of 10 ml/kg in blood volume (e.g. using a calculated blood volume of 60 ml/kg instead of an assumed blood volume of 70 ml/kg) would reduce the estimate of Fe incorporation by 12 % (i.e. from 75 to 63 %).

An inverse relationship was found between oral Fe absorption and the concentration of serum ferritin. Taking all fifteen subjects together, the correlation of ferritin with Fe absorption was significant (r^2 0·483, $P = 0{\cdot}004$, using a logarithmic function) while erythrocyte incorporation showed no significant association with ferritin (r^2 0·06). Furthermore, at 36 weeks the two subjects with the highest oral absorption (77·2 and 70·0 %) and reduced erythrocyte incorporation (68 and 73 %) had evidence of Fe insufficiency. (This was defined as a fall in mean cell volume from early pregnancy of more than 6 fl or a rise in Zn protoporphyrin above the normal range of 3 μg/g Hb, in the presence of Hb at or above 110 g/l (Barrett *et al.* 1994).) One subject had a Hb value of 110 g/l, serum ferritin 7 μg/l, Zn protoporphyrin 2·5 μg/g Hb and a fall in mean cell volume of 7·4 fl since 12 weeks gestation, while the other subject with a Hb of 111 g/l and serum ferritin 5 μg/l had a Zn protoporphyrin of 3·6 μg/g Hb and a fall in mean cell volume of 5 fl (within the normal range (Whittaker *et al.* 1996)).

Discussion

The use of the dual-isotope technique eliminates the errors that assumptions of erythrocyte incorporation may have on the calculation of Fe absorption. In order to quantify incorporation, some calculation of the total Fe mass was needed. This was achieved by a measurement of plasma volume and calculation of blood volume. In pregnancy, this measurement for individuals may differ substantially from literature-based estimates, such as blood volume being 70 ml/kg (Fomon *et al.* 1988; O'Brien *et al.* 1999) and can be derived for individual subjects from the equations we provide (see Appendix) using height as well as weight. Furthermore, the use of an individually determined figure for Fe incorporation, such as 60 %, instead of an assumed figure of 90 %, would mean that daily utilisation of Fe intake would be reduced by a third.

Whole-body counting has established that normal, non-pregnant, non-anaemic women, given 5 mg Fe whilst fasted, will absorb between 10 and 20 % of the administered dose (Heinrich, 1970; Bothwell *et al.* 1979). Some of our subjects had results slightly higher than this, which could be

explained by natural variability, the positive effect of ascorbic acid or by the low-Fe diet for the days preceding and during the test. In addition, one non-pregnant subject with high absorption was Fe depleted (serum ferritin <12 μg/l) (Bothwell *et al.* 1979).

We found the average absorption in early pregnancy of 11·8 % comparable with other studies which have measured the absorption of aqueous Fe during pregnancy (Whittaker *et al.* 1991) and our average of 59 % absorption for late pregnancy demonstrated once again the increase in Fe absorption during pregnancy. Other investigators using aqueous Fe in late pregnancy have reported 38 % absorption (dose 2–9 mg; Hahn *et al.* 1951), 51 % (dose 4–7 mg; Dyer & Brill, 1972) and 37 % (dose 5 mg; Whittaker *et al.* 1991). Studies showing low absorption in late pregnancy have used supra-physiological doses (giving 14 % absorption from 100 mg; Svanberg *et al.* 1975*b*) or used Fe in meals of low bioavailability (Svanberg *et al.* 1975*a*). Our data also suggested that absorption in early pregnancy was slightly lower than in the non-pregnant subjects, though the wide range of absorption did not support statistical significance. Nevertheless, this relationship has been suggested in other studies (Svanberg, 1975) and makes teleological sense as the Fe demand in early pregnancy is less than in non-pregnancy since menstruation is avoided (Bothwell *et al.* 1979). Pregnancy may also influence the ferrokinetics of absorbed Fe. In early pregnancy, non-pregnant or lower levels of absorption were accompanied by reduced levels of Fe incorporation into erythrocytes. While some suggest erythropoiesis is increased early in pregnancy (Howells *et al.* 1986), others have shown evidence for reduced erythropoiesis (Beguin *et al.* 1991) and the absorbed Fe may remain extramedullary, or if transferred to the marrow, not incorporated into new erythrocytes. The high demand, high absorption of late pregnancy might be associated with apparent low erythrocyte incorporation of Fe, as some of the absorbed Fe will be transferred to the fetus and not deposited in the marrow. The Fe demand of fetus and placenta in late pregnancy is estimated to be of a similar amount to the demands of the increased erythrocyte mass in mid pregnancy (Barrett *et al.* 1994) although both the amount and variation in fetal demand is difficult to quantify in individuals. The lower level of erythrocyte incorporation of Fe in late pregnancy also indicates that the ferrokinetics of late pregnancy are different from that which occurs in anaemic non-pregnant patients in whom high erythrocyte incorporation of Fe is usually found (Finch, 1970). The physiological mechanisms for our hypothesis remain uncertain, though competition for Fe utilisation or changes in Fe transport or receptor binding are possible.

Only two other studies have applied the principles of dual isotopic erythrocyte incorporation to stable isotopes. Dyer & Brill (1972) measured the Fe absorption in a group of twelve pregnant women between 23 and 38 weeks gestation using ^{58}Fe and neutron activation analysis. The single Fe isotope was used sequentially both as an indicator of erythrocyte incorporation (iv dose) and absorption (oral dose). They also used ^{50}Cr to measure plasma volumes of the subjects. Their measurement of absorption in late pregnancy agrees with ours, as does their measured erythrocyte incorporation at 64·7 (SD 12·2) %, similar to our measured levels of 63·4 and 71·0 % in early and late pregnancy respectively. This erythrocyte incorporation was significantly lower than in our non-pregnant subjects, an average of 90·2 %. This suggests that an assumed value for erythrocyte incorporation might not apply to pregnant subjects, and may lead to an overestimation of Fe utilisation by single isotope studies during pregnancy.

A recent dual-isotope study of Peruvian women in late pregnancy (O'Brien *et al.* 1999) found that unsupplemented women absorbed only an average of 12 % of a 10 mg Fe dose while Fe-supplemented women absorbed 10 % of a 60 mg dose. The response of the control women is suprising since it suggests no dose–response relationship, unlike other studies (Bothwell *et al.* 1979), and no pregnancy effect of the level we and others have found. One explanation may be that the bioavailability of the test solution was low (stored and administered without ascorbic acid) and further comparison against a reference dose and a non-pregnant control group is warranted. Fe incorporation data in this study were limited by the use of a standard blood volume formula that ignores individual variability (as noted earlier). However, the data suggest that erythrocyte incorporation was higher (and highly variable) in the control group (92 (SD 28) %) than in the supplemented groups (75 (SD 15) %). This may be part of the reason for their finding that the Hb and PCV did not differ between control and supplemented groups after an average of 17 weeks of supplementation at 60 mg/d, and had not in fact risen in the supplemented groups since the start of therapy (though ferritin was significantly raised). It would also be valuable to assess whether the response to supplementation was inversely related to the Hb status of the fetus and neonate and whether more Fe-deficient women transferred more Fe to the fetus.

In the current study and others (Barrett *et al.* 1994; O'Brien *et al.* 1999), the concentration of serum ferritin was inversely related to Fe absorption during pregnancy, and the two pregnant subjects in late pregnancy with evidence of Fe insufficiency had the highest absorption. However, the finding (Barrett *et al.* 1994) that Fe absorption doubled between 24 and 36 weeks of pregnancy while ferritin and Hb remained unchanged suggests that other factors (such as fetal demand) also drive Fe absorption up in late pregnancy. The post-absorptive serum method, which reflects the amount of Fe immediately absorbed into the body, found the amount of Fe absorbed at 36 weeks was not different in anaemic *v.* non-anaemic subjects (Barrett *et al.* 1994). As the erythrocyte incorporation method reflects also the Fe that is used by the marrow to synthesise Hb, the erythrocyte method is more likely to correlate estimates of absorption with marrow Fe stores and erythropoiesis. We suggest that anaemia developing during pregnancy may be the result of alterations in the fate of absorbed Fe, rather than just a result of poor absorption. It is possible that by applying both the post-absorptive serum method and the erythrocyte incorporation method concurrently to pregnant anaemic women, a difference in absorption results would reflect the reduced ability of that subject to incorporate absorbed Fe into Hb. Longitudinal studies of erythrocyte incorporation may help to identify the process by which Fe-deficiency anaemia develops in the presence of good dietary intake and increased oral absorption.

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APPENDIX

Calculation of plasma volume and blood volume

We present here, for the benefit of future investigators, Tables enabling calculation of plasma volume in relation to maternal height, weight and haemoglobin. Estimates of plasma volume were derived in a serial study using Evans Blue dye dilution on fifty-six women (without Fe supplementation) and the data on changes in the mean values of plasma volume and haematology have been published (Whittaker & Lind, 1993; Whittaker *et al.* 1996). Regression analysis showed height and haemoglobin were significant variables as well as weight. Comparison of our equation in Table A2 for non-pregnant women with that published in a reference text (Documenta Geigy, 1981) gave a mean difference between estimates of 204 (SD 72) ml (*n* 64). We also show the changes in blood volume in our subjects expressed in terms of body weight.

Table A1. Estimation of plasma volume during pregnancy from maternal height, weight and haemoglobin*

		Time from last menstrual period (weeks)						
	Non-pregnant	7	12	20	28	36	38	12 weeks post-natal
B1	18·9	18·3	24·6	25·2	27·7	29·3	24·2	27·6
B2	14·9	−1·3	8·6	9·8	10·2	13·6	12·4	7·6
B3	−82·0	−145	−125	−185	−186	−247	−68·8	−95·4
A	−482	+1412	−312	+532	+378	+605	−463	−1313
R	0·710	0·502	0·586	0·667	0·610	0·659	0·402	0·664
PV (mean value, ml)	2378	2472	2667	3108	3541	3646	3620	2409
CV of estimate (%)	8·9	10·6	9·2	8·1	9·1	10·4	12·3	9·1

PV, plasma volume.
* PV (ml) = (Ht (cm) × B1) + (Wt (kg) × B2) + (Hb (g/dl) × B3) + A, where Ht is the height and Hb is the haemoglobin.

Table A2. Estimation of plasma volume during pregnancy from maternal height and weight if haemoglobin data are unavailable or iron supplementation is given*

		Time from last menstrual period (weeks)						
	Non-pregnant	7	12	20	28	36	38	12 weeks post-natal
B1	20·2	21·8	24·2	27·9	25·6	36·1	22·9	28·3
B2	14·9	−4·7	5·9	1·7	5·8	11·0	11·8	6·8
A	−1793	−815	−1648	−1557	−1040	−3029	−982	−2637
R	0·676	0·354	0·490	0·455	0·392	0·478	0·382	0·598
CV of estimate (%)	9·3	11·3	9·7	9·6	10·5	12·0	12·2	9·5

* PV (ml) = (Ht (cm) × B1) + (Wt (kg) × B2) + A, where PV is the plasma volume and Ht is the height.

Table A3. Blood volume during pregnancy expressed per body weight (ml/kg)

		Time from last menstrual period (weeks)						
	Non-pregnant	7	12	20	28	36	38	12 weeks post-natal
Mean	62·7	63·2	65·4	70·8	73·5	72·3	71·4	61·2
SD	6·4	9·5	8·0	8·0	8·5	8·6	8·7	6·9
Minimum	50·2	44·6	49·7	55·1	56·7	55·5	54·3	47·6
Maximum	75·3	81·7	81·1	86·5	90·2	89·1	88·6	74·8
n	50	52	56	53	52	51	45	56