

Day-to-day variation in iron-status measures in young iron-deplete women

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In intervention and observational studies, it is necessary to determine the number of blood samples required to estimate the true value of Fe-status measures. The aim of the present study was to determine the number of days for blood sampling required in order to measure the 'true value' of five Fe-status parameters in young Fe-depleted women and to investigate the effect of menstrual cycle on these measures. Twelve women (aged 23–30 years), non-anaemic but with low Fe stores, participated in the study. Venous blood samples were collected under standardised conditions on fifteen non-consecutive days during a 5-week period. All blood samples were analysed for Hb, serum ferritin (SF), serum transferrin receptors (sTfR), red blood cell volume distribution width (RDW) and reticulocytes (RET), and body Fe stores were calculated as the ratio between sTfR and SF. No systematic changes were found in the investigated parameters during the study. When analytical variations were accounted for, the day-to-day variations (CV%) were as follows: Hb 2.9%, SF 8.2%, RET 26.0%, RDW 2.4% and sTfR 8.1%. Calculating the 'true value' with a 5% significance level and 80% power showed that one blood sample was sufficient for Hb, SF, sTfR and RDW, whereas seven blood-sampling days were needed for RET. In this study, no significant differences in Fe status were found across the menstrual cycle. The conclusions from this study are valid for studies conducted under similar strict conditions.

Iron-status measures: Day-to-day variability: Menstrual cycle

Worldwide, Fe is the micronutrient most commonly found to be deficient, with children and women of reproductive age being the most at-risk population groups (van de Vijver *et al.* 1999; United Nations, NCC/SCN/2000). Fe deficiency is mainly ascribed to a low Fe intake and/or a low bioavailability of dietary Fe, leading to insufficient replenishment of the basal Fe loss, such as menstrual bleeding (Hallberg, 1992; Andrews, 1999; Hallberg *et al.* 2000; United Nations, NCC/SCN/2000). Thus, in studies on Fe deficiency and related problems, individuals in specific sex and age groups are used.

In assessing Fe status, it is important that the most reliable Fe-status measures are used. The optimum number of blood samples per individual must be collected to take into consideration biological (individual) day-to-day variation in the blood concentration of a parameter as well as analytical variation. In human studies, the assessment of Fe status is often based on a single blood sample. If the day-to-day variation is large, the determination of Fe status may be misinterpreted. On the other hand, it is important to limit the number of blood samples to the minimum required in order to reduce Fe loss, discomfort, cost of analysis and time spent.

Previous methodological studies have suggested that 1–3 blood-sampling days over a time period of 14–31 d may be required for estimating serum ferritin (SF) concentration (Borel *et al.* 1991; Alhuwalia *et al.* 1993; Cooper & Zlotkin, 1996; Lammi-Keefe *et al.* 1996), a frequently used measure of Fe status. Estimation of the true value of other Fe-status measures,

such as Hb and serum transferrin receptors (sTfR), has been suggested to require one (Borel *et al.* 1991; Lammi-Keefe *et al.* 1996) and one or two sampling days (Alhuwalia *et al.* 1993; Cooper & Zlotkin, 1996; Lammi-Keefe *et al.* 1996), respectively. As no published studies have focused on Fe-depleted young women, there is a lack of knowledge of the required number of sampling days in this subgroup.

Previous studies have suggested that different factors may have confounding effects on SF. Confounders such as infection (Hulthén *et al.* 1998), alcohol consumption (Strain & Thompson, 1991), physical activity (Fry *et al.* 1992) and diurnal cycle (Lammi-Keefe *et al.* 1996) have been noted. Smoking appears to be associated with increased levels of Hb (Hutter *et al.* 2001). Fe loss caused by menstrual bleeding may also be a possible confounder of the level of Fe-status measures. In an observational study, the concentrations of Hb and SF were reported to be significantly reduced during the bleeding period compared with the luteal phase (Kim *et al.* 1993). However, standardisation of the above confounding factors was not always taken into account in the design of the methodological studies.

The aim of the present study was to determine the required number of days for blood sampling over a time period of 5 weeks in order to measure the 'true value' of five different Fe-status measures in young non-anaemic but Fe-depleted women. A specific objective was to investigate the effect of the menstrual cycle on the measurements in a 5-week study.

Abbreviations: α -ACT, alpha-antichymotrypsin; CV_{day}, day-to-day variation; CV_{biol}, biological variation; CV_{anal}, analytical variation; RDW, red cell distribution width; RET, reticulocytes; SF, serum ferritin; sTfR, serum transferrin receptors.

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Subjects and methods

Subjects

The inclusion criteria for the subjects in the present study required that they were Fe-depleted but non-anaemic women with an SF level between 12 and 30 $\mu\text{g/l}$ and an Hb $\geq 119 \text{ g/l}$. Forty-one healthy young women were screened for concentrations of SF and blood Hb (SF, geometric mean 20.5 (95% CI 16.5, 24.6) $\mu\text{g/l}$; blood Hb, mean 125.7 (SD 8.1) g/l). Thirteen women met the inclusion criteria but only twelve completed the study as one subject was excluded owing to medication with penicillin during the study period. The study group therefore consisted of twelve healthy women between 23 and 30 years of age who were free from chronic and inflammatory diseases known to affect the level of SF, on the basis of self-reporting and screening for acute-phase protein α -antichymotrypsin (α -ACT) in serum, using values above 0.6 g/l to indicate an immunological reaction. The subjects were of normal weight (BMI mean 20.6 (SD 1.4) kg/m^2), non-pregnant, non-lactating, non-smokers and non-athletic. Furthermore, the use of any kind of medication, including oral contraceptives and vitamin/mineral supplements, 2 months before and during the study was not allowed. In addition, donation of blood 6 months before and during the study was not allowed. At baseline, the concentrations of SF and blood Hb were geometric mean 21.0 (95% CI 17.2, 25.5) $\mu\text{g/l}$ and mean 130.5 (SD 8.1) g/l , respectively. All subjects gave their written consent after having received verbal and written information about the study. The Municipal Ethical Committee of Copenhagen and Frederiksberg approved the study as being in accordance with the Helsinki II Declaration (KF-01-238/98).

Experimental protocol

Blood samples were collected between 08.00 and 09.00 on fifteen non-consecutive days (typically Monday, Wednesday and Friday) in a 5-week period. All subjects registered the timing and duration of their menstrual bleeding and any irregularities during the study. The menstrual cycle was monitored by measuring the level of luteinising hormone (LH) in each blood sample. All blood samples were taken under the same standardised conditions. The subjects were instructed to fast for 10 h before the blood collection and were only allowed to drink a half-litre of tap water during this period. The subjects abstained from medication and alcohol for 24 h and did not exercise during the last 36 h before each blood collection. As automatic analytical methods were used in the present study, all Fe-status measures were determined in a single measure of a blood or serum sample from each of the fifteen blood sampling days.

The blood samples were drawn, after 10 min of supine rest, from an antecubital vein made visible by the temporary use of a tourniquet, into a 2 ml vacutainer with 7.5% 0.04 ml EDTA (Benson Dickenson, NJ, USA) and a 5 ml vacutainer with no anticoagulant (Benson Dickenson). Samples were all transported on ice to the laboratory and processed within 1 h of collection.

The blood samples with EDTA anticoagulant were analysed for Hb and red cell distribution width (RDW) by blood cell counting on a Sysmex KX-21 (Sysmex Automated Hematology Analyzer Corporation, Kobe, Japan). Before analysis, each sample was gently mixed for a few minutes. The intra- and inter-assay

variations for Hb were 0.7% and 1.1%, respectively, and for RDW 1.1% and 0.8%, respectively. After analysis of Hb and RDW, the blood samples with EDTA anticoagulant were stored at 5°C for a maximum of 3 d, during which time they were analysed for the concentration of reticulocytes (RET). The analysis of RET was carried out on a Pentra 120 II system (ABX Diagnostics; Montpellier, France) by a flow cytometric method. The intra- and inter-assay variations for RET were 10.2% and 8.3%, respectively.

For the blood samples without anticoagulant, serum was separated within 1 h after collection by centrifugation at 3000 g for 15 min at 20°C, divided into 200–400 μl aliquots of serum in cryo tubes and stored at -20°C for the later analysis of SF, LH, α -ACT and sTfR.

SF was determined by a solid-phase fluoroimmunoassay method on an AutoDELFIA system (1235-514; Wallac Oy, Finland) with an AutoDELFIA ferritin kit (B069-101). The intra- and inter-assay variations for SF were 2.1% and 5.5%, respectively. The analysis was standardised against a ferritin standard obtained from the WHO International Laboratory for Biological Standards Ferritin, Human spleen for immunoassay (80/578) (National Institute of Biological Standards and Controls, South Mimms, Hertfordshire, UK).

The acute-phase protein α -ACT was determined by an immunoturbidimetric method on a Cobas Miraplus system (Roche Diagnostic Systems, F. Hoffmann-La Roche Ltd, Basel, Switzerland) with intra- and inter-assay variations for α -ACT of 2.5% and 3.0%, respectively. Reagents were obtained from DAKO A/S (Glostrup, Denmark).

The concentration of sTfR was determined by an immunoturbidimetric assay (IDeA sTfR-IT; Orion Diagnostica, Espoo, Finland). The intra- and inter-assay variations for sTfR were 2.9% and 5.5%, respectively.

Luteinising hormone was determined by a solid-phase fluoroimmunoassay method on an AutoDELFIA system (1235-514) with an AutoDELFIA human luteinising hormone kit (B031-101). The intra-assay variation for luteinising hormone was 3.2%. All fifteen serum samples from each subject were analysed in a single batch to eliminate batch-to-batch analytical variation. In total, 105 ml blood was drawn from each subject.

Statistical analyses

Except for SF, the results are given as the mean \pm SE. The SF results are given as the median and 95% CI. Statistical analyses were performed with SAS 8.2 (SAS Institute, Cary, NC, USA), and the significance level was set at $P < 0.05$. Prior to the statistical analyses, all data were tested for normality by the Shapiro–Wilk W test and variance homogeneity. It was necessary to transform the results logarithmically for SF. The results for sTfR from one subject were excluded as they were abnormally high.

The total day-to-day variation (σ_{day}^2) was defined as the sum of the biological variation (σ_{biol}^2) and the analytical variation (σ_{anal}^2), i.e. $\sigma_{\text{day}}^2 = \sigma_{\text{biol}}^2 + \sigma_{\text{anal}}^2$ (Borel *et al.* 1991). σ_{biol}^2 was estimated by a general linear mixed model with repeated measurements. Subject and days within subjects were included in the model as random variables.

σ_{anal}^2 was estimated by the sum of the intra- and inter-assay variations of a known control sample with a concentration within the range of the present study. The required number of blood sampling days (one blood collection per sampling day)

for the 'true average value' of the parameter, D , with a 5% significance level and 80% power was determined by using the modified equation described by Basiotis *et al.* (1987):

$$D = (Z^2 \times \sigma_{\text{day}}^2) / (A^2 \times (\text{total mean of the analyte}^2))$$

where Z is 1.96 for a level of significance set at 0.05 and A is the level of accuracy ($A = 0.2$). This means that D is the required number of days (based on one blood collection per day) to estimate the true average value of the Fe-status measure for the individual, as the value obtained is within 20% of the true serum concentration 95% of the time.

The level of body Fe stores as estimated after Cook *et al.* (2003) as the ratio between sTfR and SF as given:

$$\text{BI (mg/kgbody weight)} = -(\log(\text{sTfR/SF}) - 2.8229) - 0.1207$$

The menstrual cycle was divided into the menstrual, follicular and luteal phases. The luteal phase included ovulation estimated by the luteinising hormone level. Differences between phases were tested by using a general linear mixed model with repeated measurements. The autocorrelation between repeated measurements was taken into account by using an autoregressive model. *Post hoc* comparisons were made, with Turkey–Kramer adjustment of significance levels for the pairwise comparison, using an unpaired *t*-test.

Results

There was no indication of infection in the analysed blood samples as all serum α -ACT values were below the cut-off value of 0.6 g/l.

The mean variance components (σ_{biol}^2 and σ_{anal}^2) and biological, analytical and total day-to-day CV for each investigated Fe-status measure are presented in Table 1. For all variables, the CV_{biol} were higher than the corresponding CV_{anal} . The CV_{day} for each Fe-status measure varied considerably, ranging from 2.4% to 26.0%. No indication was found of a systematic change in Fe-status parameters or in Fe stores during the study as no significant effect of time and blood sampling day on the investigated parameters or body iron stores was found (Fig. 1).

The number of days required for estimating the true average value of the investigated Fe-status measures with a 5% significance level and 80% power was one blood sampling day for Hb, SF, sTfR and RDW, and seven blood sampling days for RET. No significant effect of menstrual cycle was found for any of the Fe-status measures (Table 2).

Discussion

In the present study, we found that one blood sample day was sufficient to determine the 'true value' of Fe status as determined by

the indicators Hb, SF, sTfR and RDW, but not by RET, in strictly standardised studies with young women. This finding is important with regard to clinical and nutritional intervention studies, as it minimises the number of blood collections, the subject's discomfort, the disruption of the subject's normal life, study costs and time for analysis. Furthermore, the results indicated that the Fe-status measures in young Fe-depleted women did not change significantly during the menstrual cycle.

In most studies, investigators report the intra-individual variation in Fe-status measures when summing up study results. Intra-individual variation is the sum of biological and analytical variation, which determines the number of blood sampling days required for the assessment of the 'true value' of Fe status. The present estimation of the required number of blood sampling days and day-to-day variation was based on the concept previously established in a study with ten young men and women (Borel *et al.* 1991). A different statistical approach was, however, used in the present study. In the study by Borel *et al.* (1991), analytical variation was estimated by the component of variation of either duplicate or triplicate measures of the same blood sample. In the present study, all variables were assessed in a single measure as automatic analytical methods were used. Analytical variation was therefore calculated as the product of intra- and inter-assay variations of the analysis. With the continuing improvement of methodology, the influence of analytical variation is, however, decreasing, and biological variation is the main contributor to the total day-to-day variation. It is therefore of more use to clinicians and others to emphasise the total determined variation of Fe-status measures.

Other studies have determined the biological and intra-individual (day-to-day) variations of different Fe-status measures in ten young men and eleven women (Cooper & Zlotkin, 1996) and in groups of ten elderly women (Alhuwalia *et al.* 1993; Lammi-Keefe *et al.* 1996). As in the above studies, we also found that CV_{biol} was the major source of intra-individual variation and was considerably higher than CV_{anal} . We found that the biological variation for Hb (2.7%), SF (6.7%) and sTfR (6.9%) was considerably lower than that found by Borel *et al.* (1991; Hb 4.2%) and Cooper & Zlotkin (1996; SF 26.7%, sTfR 12.2%) with young cohorts. As biological variation is the major contributor to the total day-to-day variation, the intra-individual variations were lower in the present study (Hb 2.9%, SF 8.2%, sTfR 8.1%) than in the studies by Borel *et al.* (1991; Hb 4.4%) and Cooper & Zlotkin (1996; SF 27.4%, sTfR 12.9%). The lower day-to-day variations for Hb, SF and sTfR in the present study were probably due to a stricter standardised study design and a more homogenous group of subjects.

Table 1. Mean (geometric mean for SF), variance components, biological day-to-day variation (CV_{biol}), analytical variation (CV_{anal}) and total day-to-day variation (CV_{day}) of six Fe-status measures

	Mean	σ_{biol}^2	σ_{anal}^2	CV_{biol} (%)	CV_{anal} (%)	CV_{day} (%)
Hb (g/l blood)	125.6	11.3	2.0	2.7	1.1	2.9
SF ($\mu\text{g/l}$)	21.2	2.0	1.0	6.7	4.7	8.2
sTfR (mg/l)	1.7	6.5×10^{-3}	2.5×10^{-3}	6.9	4.3	8.1
Red blood cell volume distribution width (fl blood)	43.0	0.75	0.29	2.0	1.3	2.4
Reticulocytes ($10^9/\text{l}$ blood)	41.2	82.3	32.5	22.1	13.9	26.0
Body Fe stores (sTfR/SF)	8.4	1.0	–	12.3	–	–

SF, serum ferritin; sTfR, serum transferrin receptor; σ_{biol}^2 , biological variance component; σ_{anal}^2 , analytical variance component. n 12 subjects, except for sTfR and body Fe stores (n 11).

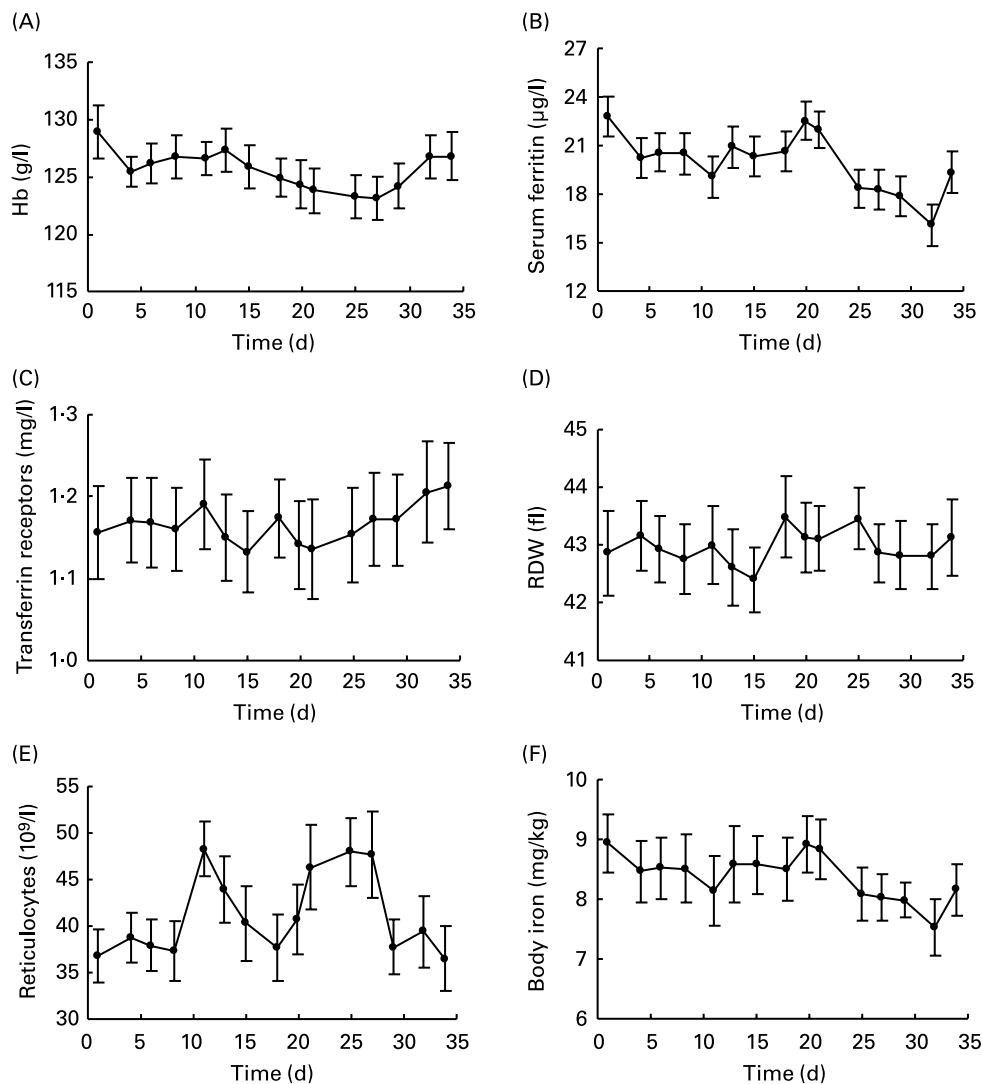


Fig. 1. Concentrations of the investigated Fe-status measures in the study group (*n* 12 except for sTfR and body Fe stores (*n* 11)) during the 5-week study period. (A) Hb, mean 125.6 (SE 1.9) g/l blood; (B) serum ferritin, geometric mean 19.9 (95% CI 19.7, 20.1) µg/l; (C) serum transferrin receptors, mean 1.2 (SE 0.05) mg/l; (D) red blood cell distribution width (RDW), mean 43.0 (SE 0.6 fl) blood; (E) reticulocytes, mean 41.2 (SE 3.5) × 10⁹/l blood; and (F) body Fe stores (serum transferrin receptors/serum ferritin ratio), mean 8.4 (SE 0.5) mg/kg.

The low day-to-day variation for Hb (CV 2.9%) resulted in only one blood sampling day being necessary to obtain the ‘true value’. This finding can possibly be explained by the fact that more than 65% of the Fe in the body is found in Hb (Hallberg, 1992). Small intra-individual changes in Hb cannot

therefore easily be detected, and one blood-sampling day is sufficient to determine the true level of Fe bound as Hb for an individual. Hb is considered to be a valid indicator of Fe-deficiency anaemia (Cook, 1999) and should always be included in the determination of Fe status, supported by one or more sensitive

Table 2. Levels of six Fe-status measures during the menstrual, follicular and luteal phases (Geometric mean and 95% CI)

	Menstrual phase	Follicular phase	Luteal phase
Hb (g/l blood)	124.1 (122.0, 126.3)	126.5 (125.0, 128.0)	125.3 (123.9, 126.7)
SF (µg/l)	20.7 (18.2, 23.4)	20.3 (18.6, 21.9)	19.1 (17.4, 20.9)
sTfR (mg/l)	1.17 (1.11, 1.24)	1.16 (1.12, 1.18)	1.14 (1.10, 1.18)
Red blood cell volume distribution width (fl blood)	43.2 (42.6, 43.9)	42.8 (42.4, 43.3)	42.8 (42.4, 43.3)
Reticulocytes (10 ⁹ /l blood)	38.6 (34.7, 42.7)	41.4 (38.9, 44.7)	37.4 (34.7, 39.8)
Body Fe stores (sTfR/SF)	8.3 (7.4, 9.2)	8.5 (7.7, 9.4)	8.3 (7.5, 9.2)

SF, serum ferritin; sTfR, serum transferrin receptor.
n 12 subjects, except for sTfR and body Fe stores (*n* 11).

parameters. SF is positively correlated with the size of body Fe stores (Lipschitz *et al.* 1974) and is therefore negatively correlated to the percentage of Fe absorption (Magnusson *et al.* 1981). SF is considered to be the best indicator of Fe status and is also the most widely used (Hulthén *et al.* 1998). Unfortunately, SF is highly influenced by factors such as infection (Ferguson *et al.* 1992; Petterson *et al.* 1994; Hulthén *et al.* 1998), and probably also alcohol (Leggert *et al.* 1990; Strain & Thompson, 1991; Milman *et al.* 2000), physical activity (Fry *et al.* 1992) and seasonal variation (Maes *et al.* 1997). SF, together with Hb, is often used in assessing the Fe and health status of individuals: SF gives a good indication of the size of the Fe stores in the absence of infection and inflammation, whereas Hb denotes the amount of Fe bound in erythrocytes and thereby facilitates the detection of anaemia.

In recent decades, sTfR has become a more commonly used Fe-status measure and is considered to be the single most sensitive indicator of functional Fe depletion (Baynes, 1996). Furthermore, sTfR is less sensitive to infection (Ferguson *et al.* 1992; Petterson *et al.* 1994) and seasonal variation (Maes *et al.* 1997). sTfR is negatively correlated to the size of the Fe stores and seems to respond earlier to Fe deficiency than do mean corpuscular volume, mean cell Hb and RDW (Baynes 1994). sTfR is a recommendable 'supplement' to the measurement of SF. In the present study, sTfR was analysed by a monoclonal immunoturbidimetric method, which may explain the lower average level of 1.7 mg/l found, compared with the 3.9 mg/l found by Cooper & Zlotkin (1996) using an ELISA method. Despite the difference in analytical methods, both studies showed that one sampling day was sufficient to determine the 'true value' of sTfR. A new approach was adapted to determine the size of body iron stores/kg, estimating body iron stores as the ratio between sTfR and SF, as suggested by Cook *et al.* (2003). When analysing this approach, there was no indication of a systematic depletion of body Fe stores over time (Fig. 1).

Inconsistent results have been found regarding the usefulness of RDW in determining Fe status (Baynes *et al.* 1987; Docci *et al.* 1989; Mahu *et al.* 1990; Nielsen *et al.* 1990; van Zeben *et al.* 1989). In the present study, no correlation was found between RDW and the other measures investigated (results not shown).

Finally, in theory, RET might be proposed as a useful Fe-status measure as it reflects the first circulating form of red blood cells, existing in the circulation for only 1 d (Löwenstein, 1959). It therefore provides information about the level of release of circulating Hb. However, the usefulness of RET cannot be supported in the current study owing to the large CV_{biol} and CV_{anal} determined for this measure.

In the present study, the menstrual cycle had no effect on the Fe-status measures. This was in contrast to an observational study with 1712 women (not taking oral contraceptives), in which it was found that Hb was significantly lowered during the menstrual phase compared with the follicular, luteal or late luteal phase (Kim *et al.* 1993). SF was also significantly lower during the menstrual phase than during the luteal and late luteal phases. In the present study, the lack of effect of the menstrual cycle is probably due to the limited number of subjects. The strength of the present study design was, however, that the subjects were followed throughout a menstrual cycle, which was not the case in the study by Kim *et al.* (1993).

In conclusion, the present results show that only one blood sampling day is required to determine the true value of Fe

status with a 5% significance level and 80% power using the indicators Hb, SF, sTfR and RDW in a homogenous group of young women, with the blood sampling carried out under strictly standardised conditions. Hb and SF must still be considered as required measures for the determination of Fe status as the two measures indicate the amount of Fe bound as Hb and the size of Fe stores bound primarily as ferritin, respectively. Unlike SF, sTfR seems to be an especially useful measure as it is not affected by infection or seasonal variation. The usefulness of RDW is difficult to appraise as the results of present and previous studies were inconsistent. RET cannot be recommended owing to the very large CV_{day} . The menstrual cycle had no effect on the level of the Fe-status measures determined in this study. Owing, however, to limited number of subjects, further studies are required.

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