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**EFFECTS OF MODEST IRON LOADING ON IRON INDICES IN HEALTHY
INDIVIDUALS**

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Running head: Modest iron loading in healthy individuals

23 **NEW & NOTEWORTHY**

24 *There has been an increasing interest in administering intravenous iron to patients to alter*
25 *their iron status. Here we explore various indices of iron loading and show that, in healthy*
26 *volunteers, serum ferritin provides a robust indicator of the amount of iron loaded, with a*
27 *value of 21 µg/L increase in ferritin per 100 mg of iron loaded.*

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29 **KEY WORDS**

30 Iron loading; ferritin; pulmonary circulation

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ABSTRACT

Intravenous (iv) iron administration is typically indicated in individuals who have iron deficiency refractory to oral iron. However, in certain chronic disease states, such as heart failure, it may be beneficial to administer iv iron to individuals who are not strictly iron deficient. The purpose of this study was to define a dose-response relationship between clinical indices of iron status and modest loading with iv iron in healthy, iron-replete participants. This was a double-blind, controlled study involving 18 male participants. Participants were block randomised 2:1 to the iron and saline (control) groups. Participants in the iron group received 3.75 mg/kg body weight up to a maximum of 250 mg of iv iron, once a month for six months, provided that their ferritin remained measured within the week before a dose was due $< 300 \mu\text{g/L}$ and their transferrin saturation remained $< 45\%$. Otherwise they received a saline infusion, as did the control participants. Iron indices were measured monthly during the study. The pulmonary vascular response to sustained hypoxia and total hemoglobin mass were measured before, at three months (hemoglobin mass only) and at six months, as variables that may be affected by iron loading. Serum ferritin was robustly elevated by iv iron by $0.21 \mu\text{g/L/mg}$ of iron delivered (95% CI: $0.15\text{-}0.26 \mu\text{g/L/mg}$), but the effects on all other iron indices did not reach statistical significance. The pulmonary vascular response to sustained hypoxia was significantly suppressed by iron loading at six months, but the hemoglobin mass was unaffected. We conclude that the robust effect on ferritin provides a quantitative measure for the degree of iron loading in iron-replete individuals.

INTRODUCTION

Over recent years there has been increasing recognition of the clinical importance of iron deficiency in the absence of anemia (1, 15, 26). However, determining the threshold values of various iron indices on which a diagnosis of iron deficiency can be made is not straightforward. Central to the diagnosis of iron deficiency is the serum ferritin, which if $<12\text{ }\mu\text{g/L}$ is very likely to be associated with absolute iron deficiency (5, 8). However, because ferritin is an acute phase reactant, its value may be falsely high in the presence of inflammation. Transferrin saturation may also be helpful, with a value $>20\%$ being associated with normal red cell indices and a value $<16\%$ indicating an insufficient supply of iron to the bone marrow such that anemia will eventually ensue (2).

Two, major randomised control trials of the use of iv iron in patients with both chronic heart failure and iron deficiency used a diagnostic threshold for iron deficiency very different from using a threshold ferritin of $<12\text{ }\mu\text{g/L}$ or a transferrin saturation of $<16\%$ (1, 7). These studies used a diagnostic threshold for iron deficiency using ferritin of $<100\text{ }\mu\text{g/L}$, or even a ferritin of $<300\text{ }\mu\text{g/L}$ if the transferrin saturation was $<20\%$. By way of comparison, a recent cross-sectional study of iron deficiency in a population of patients with chronic obstructive pulmonary disease revealed a median value for ferritin of $28\text{ }\mu\text{g/L}$ in the iron deficient group (diagnosed either through a transferrin saturation $<16\%$ or a soluble transferrin receptor concentration $>28.1\text{ nmol/L}$) and a median ferritin value of $80\text{ }\mu\text{g/L}$ in the iron replete group (25). This study reported a prevalence of iron deficiency of 18% and 5% for the chronic obstructive pulmonary disease and control groups, respectively. Indeed, with a diagnostic criterion for iron deficiency of a ferritin $<100\text{ }\mu\text{g/L}$, some 60% of not only the patients with chronic obstructive pulmonary disease but also healthy control participants would be diagnosed with iron deficiency. Despite the laxity of the inclusion criteria for iron deficiency in these two major randomised control trials of iv iron in chronic heart failure, it is

clear that patients benefitted from iron infusion, and furthermore that this benefit was not restricted to particular subgroups. Improvements were seen in symptoms, functional capacity (including the 6-minute walk test) and quality of life (using the Patient Global Assessment), with reductions in hospital admission (1, 26). Importantly, the definition of iron deficiency used for inclusion in the trials was extremely broad, and on which most normal individuals would be classified as iron deficient. In the subgroup analyses, it was shown that iv iron was as beneficial in patients who were not anaemic and for those for whom there was no change in haemoglobin in the weeks/ months following iv iron administration. As such, an alternative interpretation of these trial results is that it may be beneficial modestly to elevate certain patients' iron stores above their normal physiological values in chronic heart failure. If so, then it becomes important to understand the dose response relationship between additional iron load and serum values for various iron indices. The guidelines of the American College of Cardiology for the management of heart failure have recently been updated to recommend iv iron within the broad range of definition of iron deficiency used in the two major randomized trials (34).

As we write, there is no recognised mechanism for why modifying the iron pool in these patients is beneficial. However, it is well recognised that iron has many biological roles, including in energy metabolism and electron transport, and in the catalytic function of a large family (50+ members) of dioxygenase enzymes that include the HIF prolyl hydroxylases and histone demethylases but also one with many members where their functions are still to be identified (23).

The purpose of the present study was to explore this relationship in healthy participants in normal iron balance. We used a dosing regimen of 3.75 mg/kg iv iron every month for six months, provided that the individual's ferritin concentration was $< 300 \mu\text{g/L}$ and transferrin saturation was $< 45\%$. We found a robust increase in ferritin concentration of

105 ~20 µg/L for every 100 mg of iron administered, but the dose response relationship for other
106 indices was much less clear cut.
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METHODS

Participants. The study was approved by the South Central National Research Ethics Service (11/SC/0530) and performed in accordance with the general ethical principles of the Declaration of Helsinki. Participants provided written, informed consent. Eighteen healthy male participants were recruited via advertisement. For inclusion in the study, participants needed to be iron replete (ferritin 30-300 µg/L and transferrin saturation 15-45%) and have detectable physiological tricuspid regurgitation as an echocardiographic target for measuring pulmonary arterial pressure. Participants were excluded if they had abnormalities on their baseline blood tests, iron indices, any chronic medical condition, or were taking iron or nutritional supplements. Smokers and participants with recent or planned travel to altitude (>2,500 m) were also excluded.

Protocols. The study protocol is illustrated in Figure 1. Participants were block randomised 2:1 to iron (ferric carboxymaltose) or saline. Each month over a six-month period, participants underwent a blood test and then received an infusion of either saline or iron. At the beginning and end of the six-month period, participants spent a day in a purpose-built chamber to assess their responses to hypoxia. At 0, 3 and 6 months, participants underwent an assessment of their red blood cell mass using a carbon monoxide rebreathing technique.

The experiment was conducted in a double blind manner. Participants wore a blindfold during the infusion and the iron/saline infusion pump was kept out of sight as it was not possible to obtain a placebo to match the black appearance of iv iron. The investigator administering the infusions reviewed the blood results, but was not involved in any other part of the trial. The recruitment, experiments and analysis were conducted by a blinded investigator.

Iron infusion. Individuals received either placebo (0.9% m/V NaCl) or ferric carboxymaltose (Ferrinject ®, Vifor Pharma Ltd) intravenously at 3.75 mg/kg body weight. The maximum dose per infusion was 250 mg, which is 0.25 of the maximal single dose that can be administered. Participants received one dose every month for six months. Blood tests were taken each month in the week before the infusion. For participants randomised to iron, if their ferritin concentration was <300 µg/L (21), their transferrin saturation was <45% and they had a normal C-reactive protein (CRP), (21) then a further increment of 250 mg of ferric carboxymaltose at 3.75 mg/kg was given each month. Over the six-month period, the maximum total dose of iron that could be received by any individual was 1.5 g.

Blood tests. Blood samples were taken in the morning after a light breakfast every month. Hematological variables measured included a full blood count (FBC), hematocrit (Hct) and mean cell volume (MCV), together with an assessment of erythropoietin concentration (EPO) using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, United Kingdom). Indices of iron status included ferritin concentration, transferrin concentration (Tf) and transferrin saturation (Tsat) together with soluble transferrin receptor concentration (sTfR) and hepcidin by ELISA (R&D Systems and Bachem Peninsula Laboratories, respectively). Both CRP and interleukin 6 (IL-6) were measured as markers of intercurrent infection/ inflammation, the latter by ELISA (R&D Systems). All ELISAs were performed according to the manufacturer's instructions. The appropriate assay diluents without the addition of serum/plasma were used as negative controls. Positive controls supplied by the manufacturers were used where applicable.

Pulmonary vascular response to sustained isocapnic hypoxia. At the beginning and end of the six-month period, participants spent eight hours in a purpose built chamber (20) during which they were exposed to sustained isocapnic hypoxia, at an end-tidal partial pressure of oxygen of 55 mmHg and an end-tidal partial pressure of carbon dioxide set at

each individual's resting level measured in euoxia. Respired gas was sampled at a constant flow rate via a fine nasal catheter at the opening of the participant's nostrils. This was continuously analysed for the partial pressures of CO₂ and O₂, and the chamber gas composition adjusted by computer at five-minute intervals so as to maintain the end-tidal partial pressures at their desired values.

At baseline, and at every hour for which the participant was in hypoxia, pulmonary arterial systolic pressure (PASP) was measured using echocardiography by recording the maximum systolic pressure gradient across the tricuspid valve and using the modified Bernoulli equation with an estimated right atrial pressure of 5 mmHg (18, 35).

Red cell mass measurements. A closed circuit rebreathing technique was used to determine total hemoglobin as an index of red cell mass. The technique used was based on a modified Prommer protocol (19, 27). The technique involved participants rebreathing from an anaesthetic bag a mixture of 1 mL/kg of carbon monoxide in 4 L of 100% oxygen over a period of 3.5 minutes. Venous blood samples were taken before the start of the procedure, and then at 10, 12 and 15 minutes after the start of the experiment to measure carboxyhemoglobin. The change in carboxyhemoglobin between the 10-minute time point and baseline generated by the fixed molar amount of carbon monoxide added to the blood was then used to calculate total hemoglobin mass.

Statistics. Comparisons of initial values between the two groups were conducted using Student's unpaired t-tests. The remainder of the statistical analysis was conducted using linear mixed effects modelling (IBM SPSS V.20). For the analysis of blood results, participants were a random factor, group and month were fixed factors, and dose of iron was a covariate in the model. For the analysis of PASP measurements, the response to sustained hypoxia was taken as the average value for PASP over the period of 3 to 8 hours (hypoxia), where the response appeared to have plateaued, minus the starting value at hour 0 (euoxia);

182 participants were taken as a random factor, group as a fixed factor, and either the dose of iron
183 or the change in serum ferritin over the six-month period as a covariate.
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RESULTS

Baseline characteristics. Average physical characteristics for the two groups together with initial values for a number of variables in this study are given in Table 1. Values for IL-6 were negligible suggesting that neither inflammation nor inter-current illness were factors in the study. Mostly, the two groups were well matched at baseline (Table 1). However, hepcidin values were noticeably higher in the iron group and it is possible that the iron group was already slightly more iron replete than the saline group before any administration of iron.

Dosing. A dose of 250 mg iv iron was administered to each participant in the iron group if the following applied: ferritin concentration was $<300\text{ }\mu\text{g/L}$ and transferrin saturation $<45\%$. The general pattern of administration of iron is illustrated in Figure 2. Of note is that, apart from the first administration of iron, for each month there was always a number of the participants for whom one of these exclusions applied. No participant received 250 mg of iron on all six occasions (Figure 2). The lowest cumulative dose received in the study was 500 mg and the highest 1.25 g.

Hematological indices. Hematological indices are shown in Figure 3. There was no statistical effect of iron administration on any of these variables. Figure 4 shows the data for total hemoglobin mass at 0, 3 and 6 months. Iron administration had no measureable effect on the index of total red cell mass.

Indices of iron status. Indices of iron status are shown in Figures 5 and 6. There is a clear and highly significant ($p < 0.001$) effect of iron administration on ferritin concentration. From the linear mixed effects modelling, the slope of the relationship between ferritin concentration ($\mu\text{g/L}$) and iron dose (mg) is 0.21 (95% CI 0.15-0.26) $\mu\text{g/L/mg}$. As expected, transferrin concentration decreased with increasing iron dose, and transferrin saturation increased with increasing iron dose, but neither of these reached statistical significance.

Surprisingly, hepcidin concentration appeared to be unaffected by this level of iron loading. sTfR was similarly unaffected.

Pulmonary vascular response. The pulmonary vascular response to the sustained eight-hour period of hypoxia was measured at the beginning and end of the six-month iron loading period. During the hypoxic exposures, values for end-tidal PCO₂ and PO₂ together with values for arterial saturation were well matched between the two groups for both exposures (data not shown). The pulmonary vascular responses are shown in Figure 7A & B. A comparison of the pulmonary vascular responses to hypoxia between the iron and saline groups at baseline (before iv iron administration) suggests that the groups are not well matched, with the mean PASP response for the saline group being larger than for the iron group. Even allowing for this, however, the PASP responses to hypoxia appeared to be suppressed at six months in the iron group, but not in the saline group. The increment in PASP with sustained hypoxia, Δ PASP, was quantified as the difference between the average value for PASP over hours 3-8, minus the value for PASP at time zero. As shown in Figure 7C, Δ PASP was significantly suppressed by the administration of iron over the six-month period in a dose dependent manner. This effect was significant whether the iron loading was expressed in terms of overall dose ($p < 0.05$, Figure 7C) or its effect on serum ferritin concentration ($p < 0.01$, Figure 7D).

DISCUSSION

This study is the first to define a dose-response relationship for the effects of iv iron administration on indices of iron stores in individuals who are already iron replete. Other studies have been concerned with the dose of iron required to replenish stores in individuals with established iron deficiency (17, 22). In our iron replete participants, for every 100 mg of iv iron, serum ferritin concentration rose by ~ 20 µg/L.

Working in the opposite direction, a relationship between body iron stores and serum ferritin concentration in 22 normal subjects was measured in 1973 by Walters *et al* based upon serial phlebotomies (32). The relationship was one of near proportionality in which every 100 mg of iron removed from the body was associated with a fall in serum ferritin concentration of 13 µg/L. However, the iron deficiency generated by this approach will have significantly increased dietary iron absorption, which introduces some uncertainty in this number. (The investigators assumed a net dietary intake of 3 mg of iron per day in their calculations (32).) The measurement of Walters *et al* has been subsequently used extensively, apparently without further confirmation (12, 13).

Further to the change in ferritin with iron loading, we found that transferrin concentration appeared to fall and transferrin saturation to rise with added iron, but these effects were far from robust and did not reach statistical significance. Somewhat remarkably, the serum hepcidin concentration was little affected by the levels of iron infused in this study. Thus it appears that the ferritin concentration provides the best quantitative index of the level of iron loading out of the indices studied – and hence the degree of supplementation of iron stores - in individuals who are already iron replete.

Physiologically, there is a question as to whether iron supply limits, at least to some degree, red cell mass (14, 16, 31). Genetic association studies in relation to red cell indices identified a gene involved in iron regulation (*TMPRSS6*) as having the most major effect on

hemoglobin concentration. (6, 30). In the present study, we found no evidence that iron infusion increases red cell mass in iron-replete individuals. However, as iron deficiency is the single most common medical condition in the world (24), it is possible that the observed genetic association arises from the presence of a significant number of individuals who are iron deficient within the association studies. One concern with our measurements of total mass of hemoglobin is that the absolute values obtained appear overlarge. We have no clear explanation for why this is the case, although it is recognised that some overestimate of total hemoglobin will arise because of carbon monoxide binding to heme groups other than hemoglobin in the body (19).

A particular motivation for undertaking this study is the increasing recognition that modulating iron stores within the normal physiological range may have significant effects. Physiologically, this has been demonstrated most clearly by the effects of acute iv iron infusion which significantly blunts the pulmonary vascular response to sustained hypoxia (28, 29). These effects are not simply due to the presence of a foreign iron-sugar complex within the blood because they persist over many weeks (4). In the present study the iron and saline groups were not especially well-matched for their pulmonary vascular response to sustained hypoxia, with the saline group demonstrating a higher initial sensitivity. Nevertheless, the results confirm that iv iron administration has a prolonged effect of blunting the response to hypoxia in a dose-dependent manner (Figure 7). Indeed, these findings raise the possibility, within individuals, of utilizing the intensity of the pulmonary vascular response to hypoxia as a surrogate measure of iron status. The effect of modulating iron status within what could reasonably be recognised as a normal range raises the possibility that modulating iron stores in patients without iron deficiency could bring clinical benefit. Indeed, this lowering of the pulmonary vascular response to hypoxia may explain why iv iron is beneficial in non-anemic

patients with chronic heart failure, many of whom are also unlikely to be iron deficient given the inclusion criteria for these studies (1, 7).

In relation to normal fluxes of iron, we note that the average daily absorption of iron is around 1-2 mg, and that this amount is lost through the shedding of epithelial cells and hair, sweat and, in women, menstrual blood loss (11). This amounts, over a six-month period to approximately 200-400 mg. Here our minimum supplementary iv dose over 6 months was 500 mg and the maximum 1.25g. Given that our study is intentionally one in which a non-steady state is exploited to obtain a relationship between iron dose and iron indices, it provides no data by which an optimal iron store can be determined. Nevertheless, if other studies are able to define a ferritin level at which patients benefit from iron loading, then our results provide guidance of the dose of iron required to reach that level.

Hepcidin is the main hormone regulating iron availability. High hepcidin inhibits the absorption of iron from the gut and inhibits the release of iron from the liver and spleen into the circulation, thus lowering the overall availability of iron. Inflammation increases levels of hepcidin thus reducing both iron absorption and availability (11). There is some evidence to suggest that this response is beneficial during infection, at least in the case of malaria, as it may limit the supply of iron to pathogens (9, 10). However, in chronic inflammatory disease, the same response may not be beneficial, and indeed is the likely mechanism underlying the anemia of chronic disease (33). Consequently, in chronic disease with an inflammatory component, it is possible that overall iron availability is low and therefore it may be beneficial to supplement the overall iron stores so as to increase iron supply. This may be particularly true in patients with heart failure or indeed patients with chronic obstructive airways disease, who often suffer from hypoxia-induced pulmonary hypertension (3, 25).

In conclusion, this study establishes a dose-response relationship between ferritin concentration and cumulative iron dose in individuals who are not iron deficient. This may

302 help guide potential future therapies using iv iron supplementation to alter physiological iron
303 status in patients who are not formally iron deficient.
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AUTHOR CONTRIBUTIONS

N.K.B., M.K.C., H.-Y.C., S.L.H, and K.L.D. performed experiments; N.K.B., M.K.C, H.-Y.C., J.L.S., N.P., and P.A.R. analysed data; N.K.B., M.K.C.,H.-Y.C. N.P., K.L.D., and P.A.R. prepared, edited & revised manuscript; J.L.S. and K.L.D. unblinded investigators; P.A.R. conception and design of research.

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443

TABLE

Table 1. *Baseline characteristics of study participants*

Variable (normal range)	Saline group	Iron group	p value
Age (yr)	32.5 ±14.5	29.1 ±11.0	0.61
BMI (kg/m ²)	24.8 ±1.7	25.4 ±4.4	0.67
Hemoglobin (g/dL)	15.1 ±1.1	14.6 ±0.8	0.24
Hematocrit (%)	0.45 ±0.03	0.44 ±0.02	0.52
Red Blood Cell Count (cells/L x 10 ⁻¹²)	5.02 ±0.47	4.83 ±1.13	0.18
Mean Cell Hemoglobin Concentration (g/dL)	33.8 ±3.7	33.0 ±3.9	0.21
Mean Cell Volume (fL)	89.0 ±4.0	91.0 ±5.2	0.46
Erythropoietin (IU/L)	7.7 ±3.9	7.6 ±2.7	0.94
Ferritin (µg/L)	98.4 ±65.9	117.3 ±67.1	0.58
Transferrin (g/L)	2.7 ±0.3	2.6 ±0.3	0.75
Transferrin Saturation (%)	37.1 ±10.3	33.7 ±6.3	0.39
Soluble transferrin receptor	23.7 ±4.5	20.6 ±3.5	0.12
Hepcidin (µg/L)	38.3 ±21.0	81.0 ±68.1	0.15
IL- 6 (µg/L)	0.68 ±0.26	0.54 ±0.19	0.22
CRP (mg/L)	0.52 ±0.04	0.68 ±0.44	0.37

Values are mean ± SD. BMI, body mass index; IL-6, interleukin 6; CRP, C-reactive protein. P values were calculated with a two-tailed unpaired Student's t-test.

FIGURE LEGENDS

Fig. 1. Protocol for the study. Eighteen participants were recruited and block randomised to saline (n=6) or iron (n =12). Participants had a blood test and subsequent infusion of either saline or iv iron every month for the six-month period. Participants were exposed to sustained isocapnic hypoxia at the beginning and end of the six-month period.

Fig. 2. Patterns of administration of iv iron. Participants in the iron group received ferric carboxymaltose each month if their ferritin was $< 300 \mu\text{g/L}$ and their transferrin saturation was $< 45\%$. Over the six-month period, the minimum dose administered in the study was 500 mg and the maximum dose was 1.25 g. The number in the circle represents the number of participants at a given dose for that time point.

Fig. 3. Hematology results. There was no significant difference in hemoglobin concentration, hematocrit, mean cell volume (MCV) or erythropoietin concentration (EPO) between saline and iron groups. The p-value in the figure is for the interaction between group and time, as obtained from linear mixed effects modelling. Values are means and error bars are \pm SE.

Fig. 4. Hemoglobin mass over time. There was no significant change in hemoglobin mass over time for either the saline or iron groups. The p-value in the figure is for the interaction between group and time, as obtained from linear mixed effects modelling. Each individual line represents a single participant and the thicker line and error bars represent the mean \pm SE.

Fig. 5. Iron indices. (A), (C) and (E) illustrate ferritin, transferrin and transferrin saturation (mean \pm SE), respectively, for the saline group (open circles) and iron group (closed circles) across the six-month period. (B), (D), and (F) illustrate ferritin, transferrin and transferrin saturation (mean \pm SE), respectively, plotted against cumulative dose of iron for the iron

group only. There was a significant positive linear relationship between ferritin concentration and iron dose (B). There was a negative linear trend between transferrin concentration and iron dose but this did not reach statistical significance (D). There was a positive linear trend between transferrin saturation and iron dose, but this did not reach statistical significance (F).

Fig. 6. Hepcidin and soluble transferrin receptor concentration. (A), (C) show mean \pm SE for saline groups (open circles) and iron group (closed circles) across the six-month period for hepcidin concentration and soluble transferrin receptor concentration (sTfR), respectively. (B), (D) represent hepcidin and sTfR concentration plotted against cumulative dose for the iron group only. There were no statistically significant effects with dose.

Fig. 7. Pulmonary artery systolic pressure (PASP) during sustained hypoxic exposure. PASP during exposure to hypoxia in (A) the saline group and (B) the iron group at the beginning (open squares) and end (closed squares) of the six-month protocol. (C) Increment in PASP (Δ PASP) during the eight-hour chamber exposure to hypoxia plotted against cumulative dose of iron for the saline group (open circles) and iron group (closed circles). (D) Increment in PASP (Δ PASP) during the eight-hour chamber exposure to hypoxia plotted against the change in ferritin (Δ ferritin) from baseline to the end of six months. There was a statistically significant effect of both dose ($p < 0.05$) and Δ ferritin ($p < 0.01$) on Δ PASP.

FIGURE 1

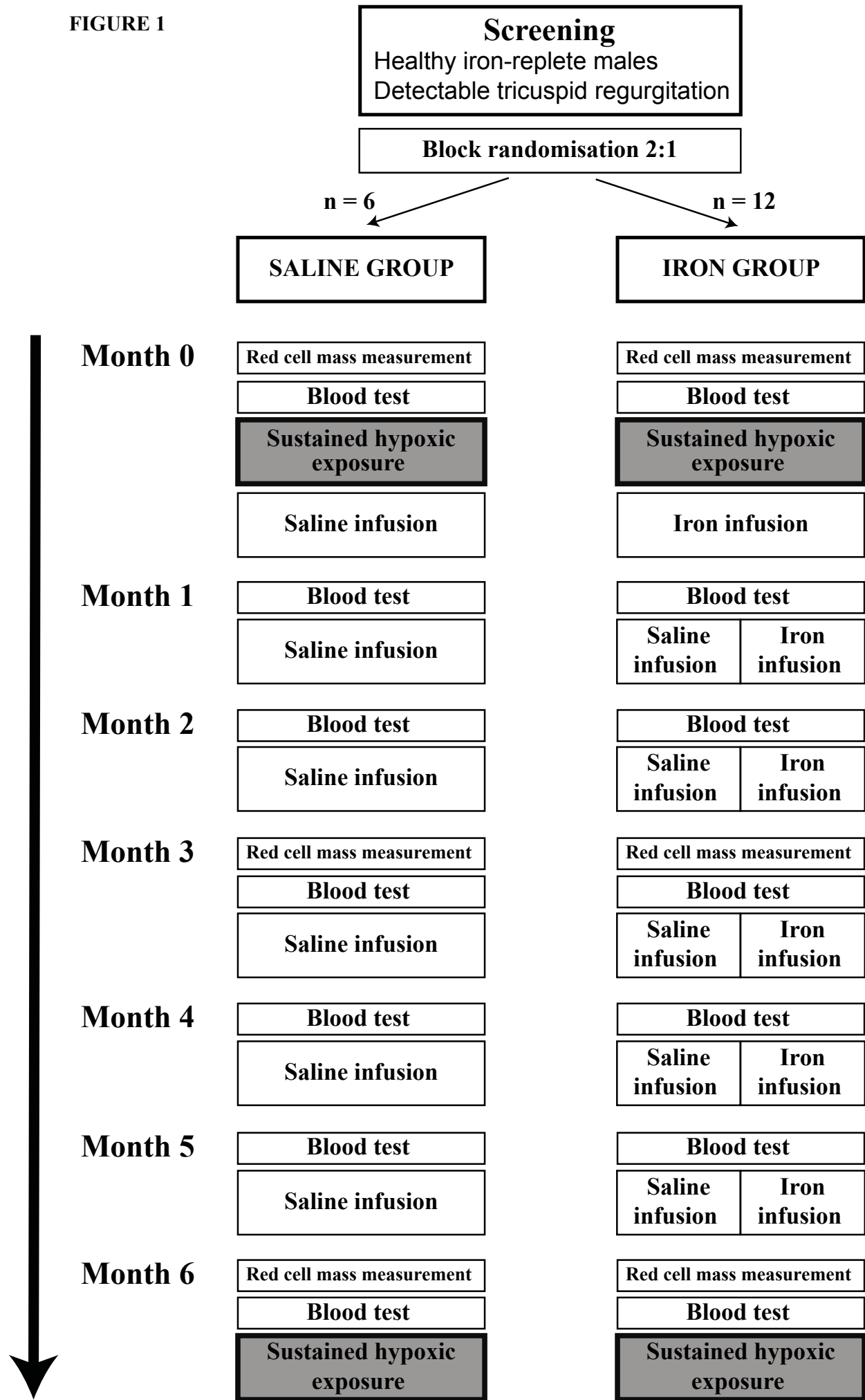


FIGURE 2

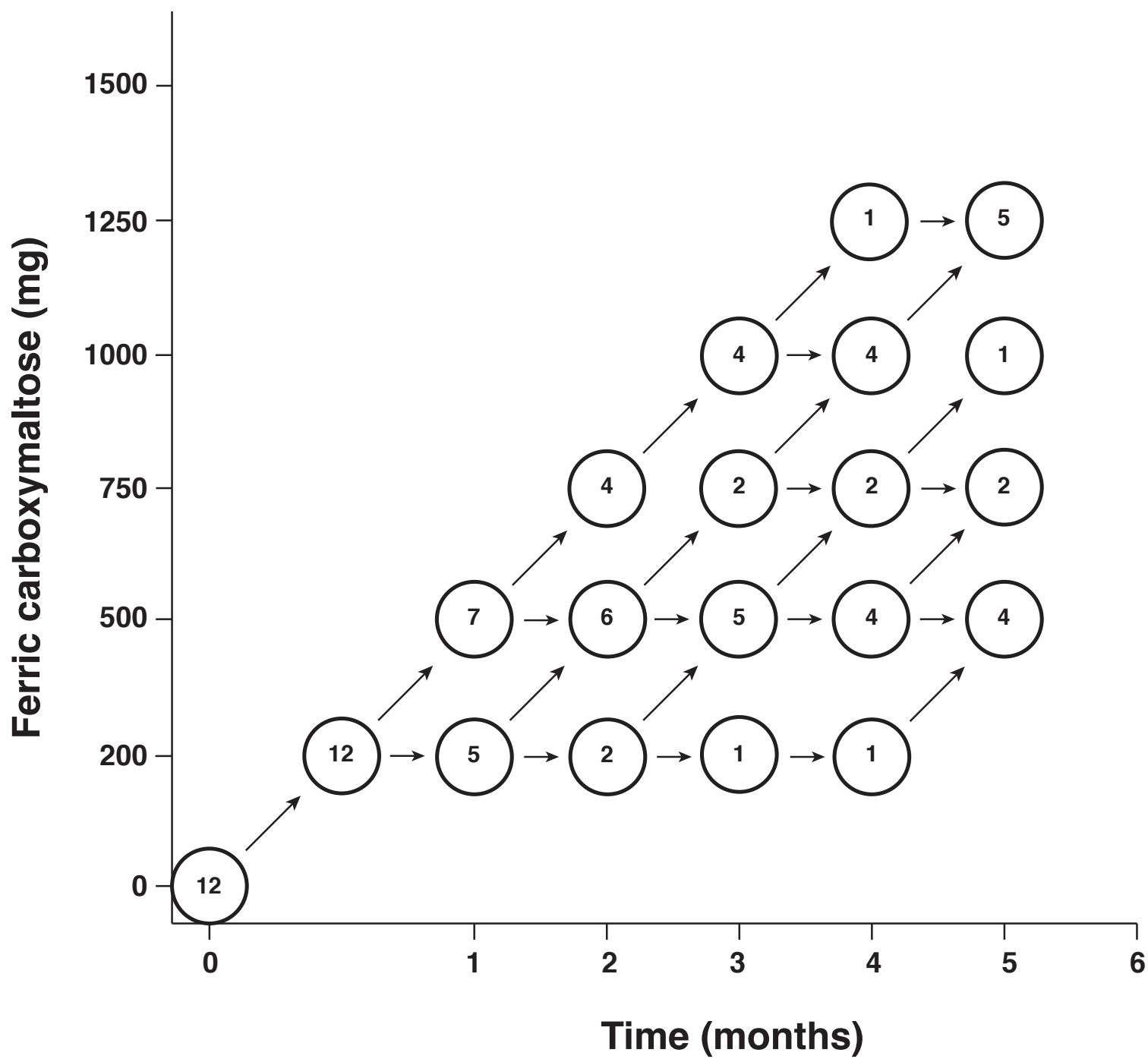


FIGURE 3

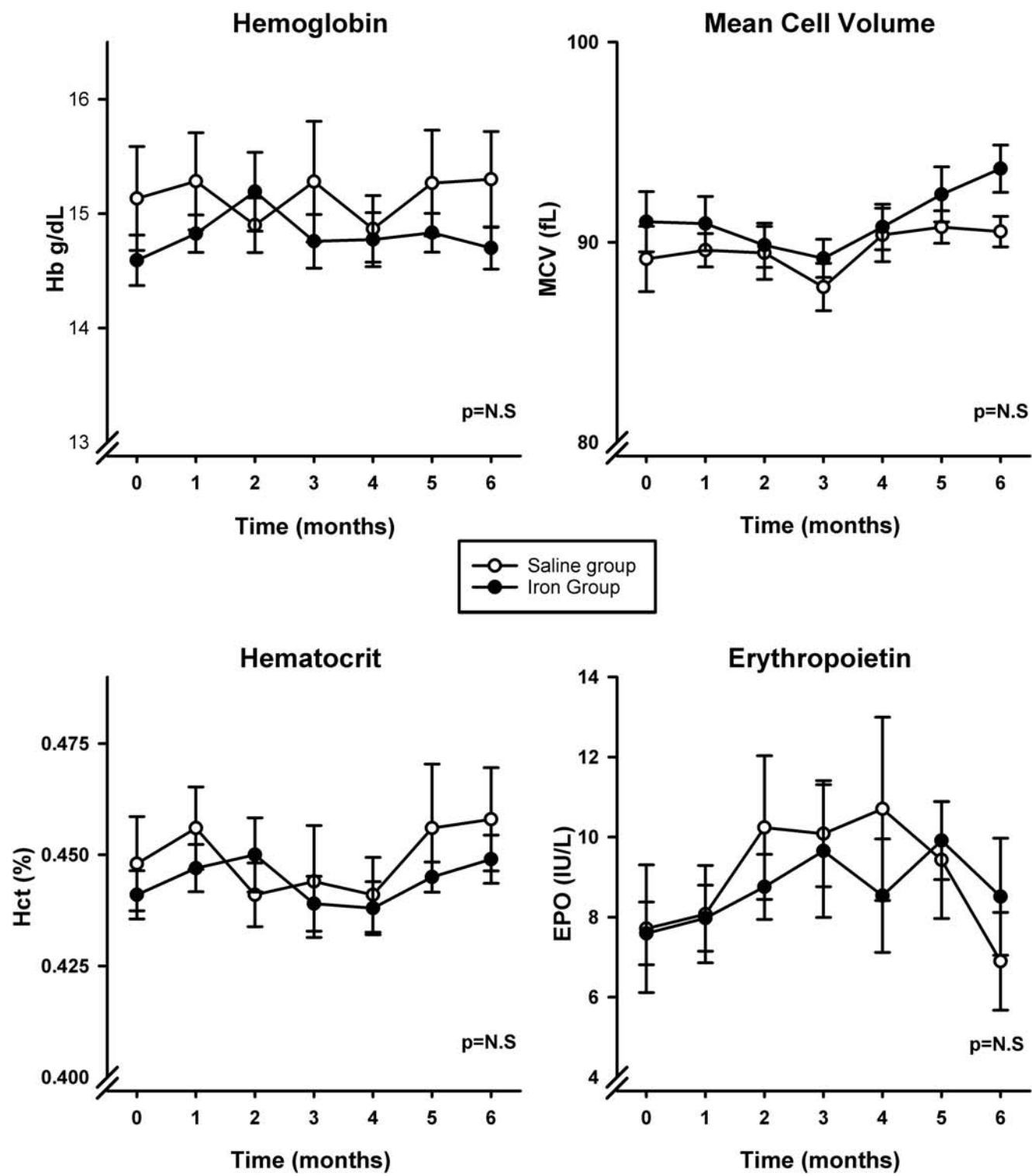


FIGURE 4

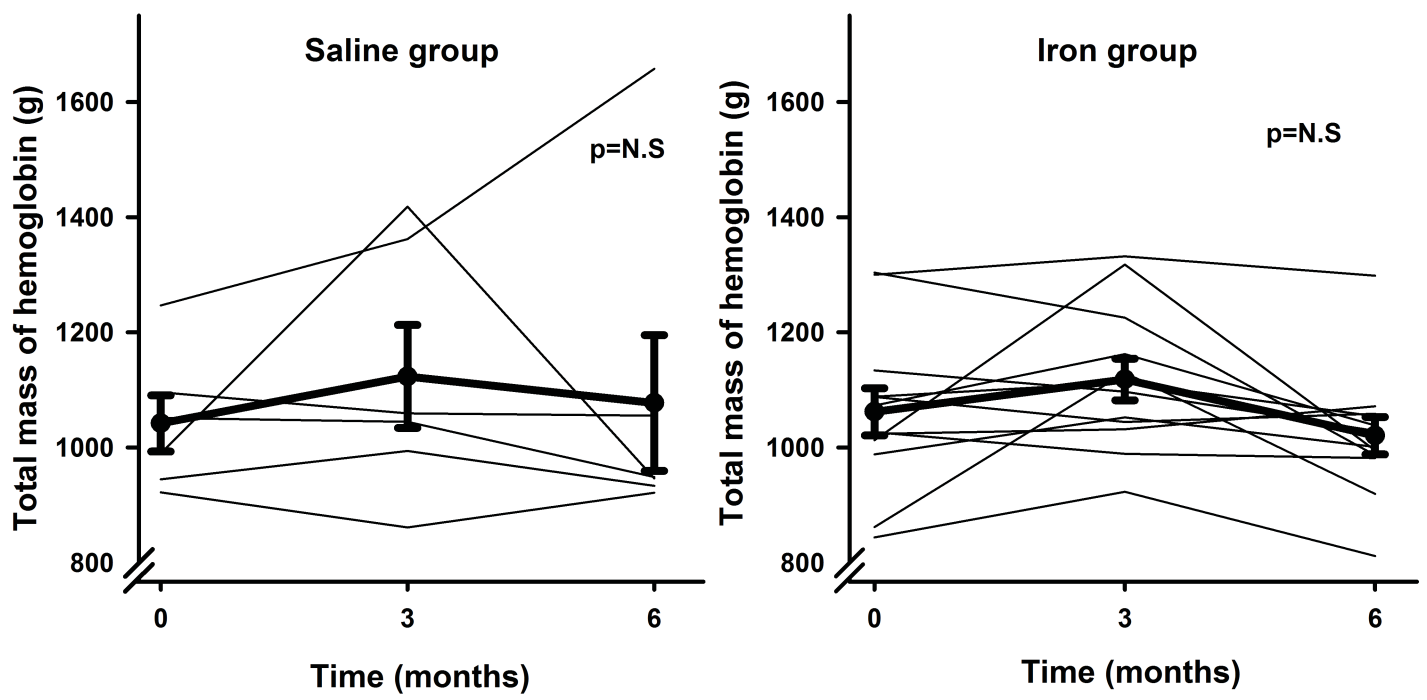


FIGURE 5

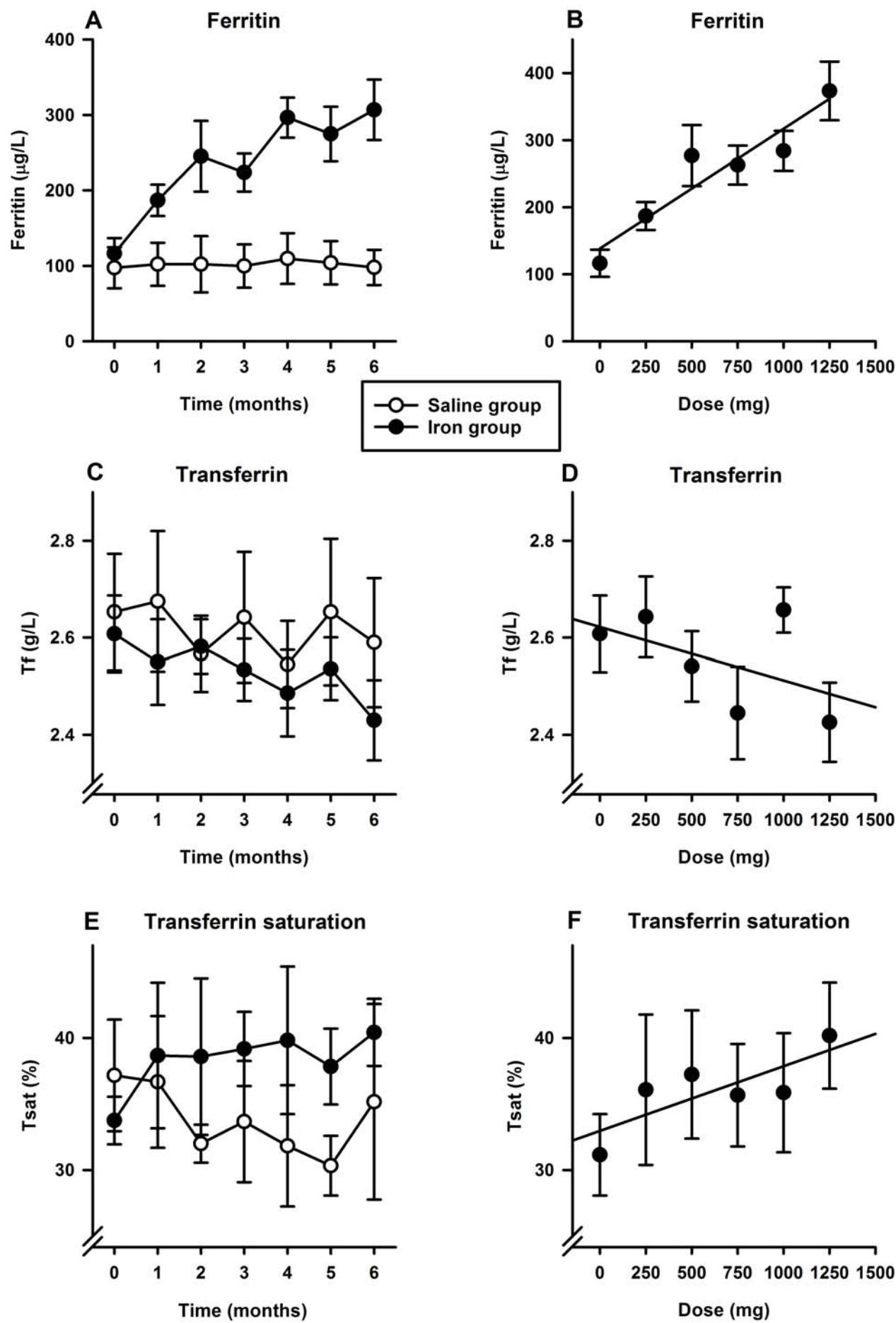


FIGURE 6

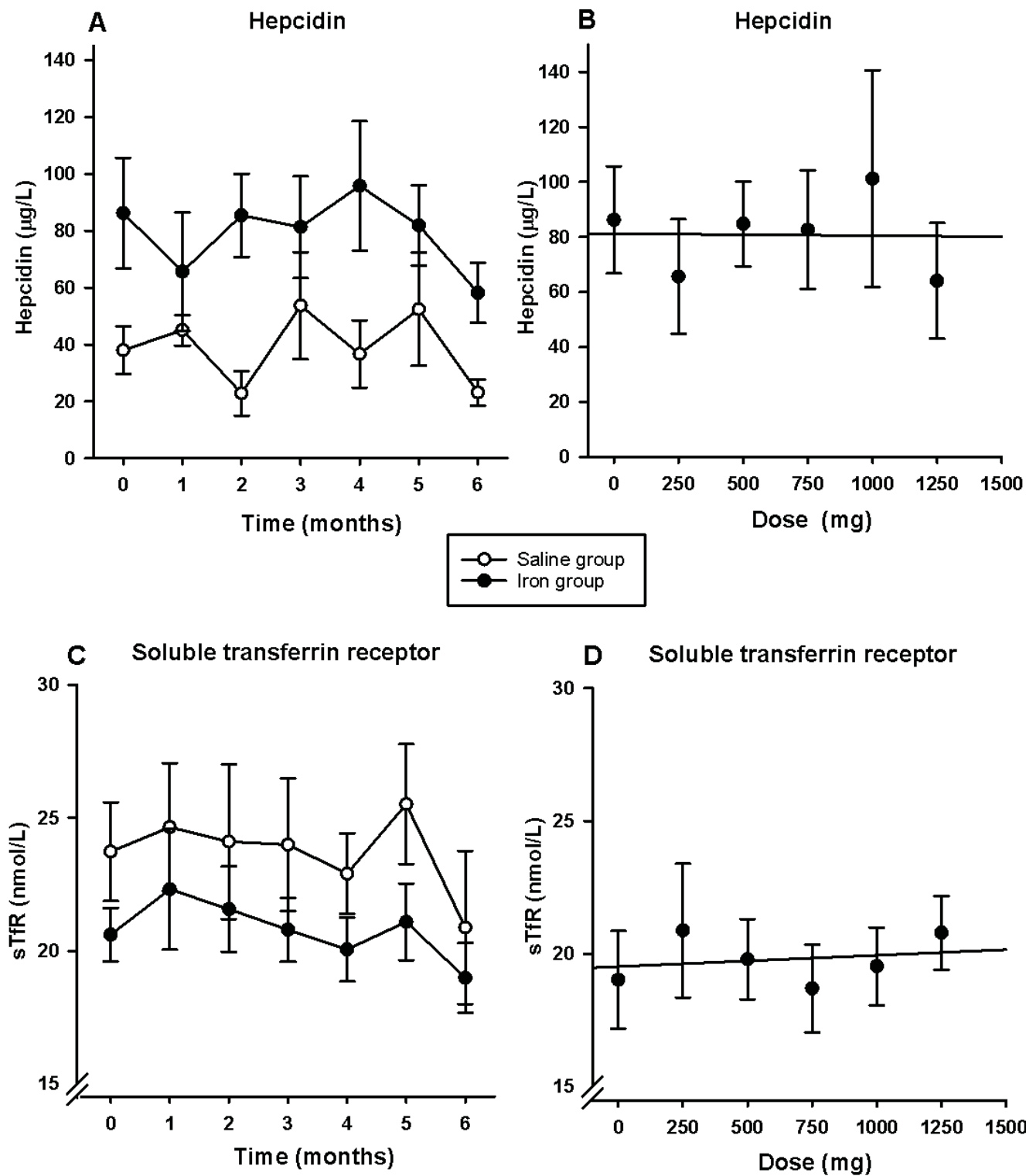


FIGURE 7