

## Iron requirements based upon iron absorption tests are poorly predicted by haematological indices in patients with inactive inflammatory bowel disease

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### Abstract

Fe deficiency and Fe-deficiency anaemia are common in patients with inflammatory bowel disease (IBD). Traditional clinical markers of Fe status can be skewed in the presence of inflammation, meaning that a patient's Fe status can be misinterpreted. Additionally, Fe absorption is known to be down-regulated in patients with active IBD. However, whether this is the case for quiescent or mildly active disease has not been formally assessed. The present study aimed to investigate the relationship between Fe absorption, Fe requirements and standard haematological indices in IBD patients without active disease. A group of twenty-nine patients with quiescent or mildly active IBD and twenty-eight control subjects undertook an Fe absorption test that measured sequential rises in serum Fe over 4 h following ingestion of 200 mg ferrous sulphate. At baseline, serum Fe, transferrin saturation, non-transferrin-bound Fe (NTBI), ferritin and soluble transferrin receptor were all measured. Thereafter (30–240 min), only serum Fe and NTBI were measured. Fe absorption did not differ between the two groups ( $P=0.9$ ; repeated-measures ANOVA). In control subjects, baseline haematological parameters predicted Fe absorption (i.e. Fe requirements), but this was not the case for patients with IBD. Fe absorption is normal in quiescent or mildly active IBD patients but standard haematological parameters do not accurately predict Fe requirements.

**Key words:** Inflammatory bowel disease: Iron absorption: Iron status: Haematological parameters: Iron deficiency

Fe deficiency (ID) and Fe-deficiency anaemia (IDA) are common in patients with inflammatory bowel disease (IBD); approximately 20–30%<sup>(1,2)</sup>. Reasons are multifactorial but protein/blood losses in the gut and low dietary Fe intakes are major drivers<sup>(3)</sup>. Fe absorption is clearly down-regulated in patients with active inflammation due to anaemia of chronic disease<sup>(4)</sup>, but it is not clear whether Fe absorption is altered in patients who are in remission. The absorption of Fe in patients with quiescent or mildly active IBD compared with healthy controls was first assessed in a pilot study, the results of which were inconclusive<sup>(5)</sup>. The outcome of a more recent study by Chermish *et al.* implies that the absorption of Fe from ferrous calcium citrate, but not iron bisglycinate, is similar in patients with quiescent Crohn's disease compared with healthy subjects<sup>(6)</sup>. However, Chermish *et al.*'s study was not

designed to compare Fe absorption in patients with IBD and healthy controls.

If Fe absorption is normal in a cohort of patients with quiescent or mildly active IBD *v.* control subjects, then further analysis can consider the relationship between Fe absorption and standard haematological parameters that are used to predict ID or Fe repletion (IR). Apart from assessing bone marrow stores, which is ethically difficult, Fe absorption probably provides the most sensitive test of Fe requirements (i.e. Fe status)<sup>(7)</sup>. Thus, standard haematological parameters that are used to predict Fe status, and may be perturbed in low-grade chronic inflammation and/or relapsing–remitting inflammation<sup>(8)</sup>, can be assessed for their predictive value or effectiveness. Hence, in the present study, both Fe absorption and its relationship to haematological parameters have been assessed in patients with IBD and control subjects.

**Abbreviations:** HBI, Harvey–Bradshaw index; IBD, inflammatory bowel disease; ID, Fe deficiency; IDA, Fe-deficiency anaemia; NTBI, non-transferrin-bound Fe; TIBC, total Fe-binding capacity.

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The method of sequential blood sampling following ingestion of ferrous sulphate was used, as this provides a direct and relevant measure of Fe absorption<sup>(9)</sup> as opposed to utilisation (e.g. erythrocyte incorporation), which may be independently perturbed in inflammatory conditions. In addition, the method of sequential blood sampling allows non-transferrin-bound Fe (NTBI) to be measured. NTBI has been proposed to occur transiently in serum, following ingestion of therapeutic supplements by Fe-deficient subjects<sup>(10,11)</sup> and even in some subjects with normal Fe stores<sup>(11)</sup>. The rationale is that the rate of absorption is too great for transferrin to completely bind the incoming Fe, and thus a small proportion binds to albumin or citrate, or even undergoes partial hydrolysis forming polyhydroxy Fe species<sup>(12)</sup>. In such forms (i.e. not bound to transferrin), Fe may be prone to redox cycling and therefore promote oxidative stress within the circulation<sup>(13,14)</sup>. It has been proposed that the antioxidant capacity of the mucosa and the circulation is depleted in IBD<sup>(15–17)</sup> such that the formation of NTBI could induce oxidative damage more readily than in control subjects.

The present study aimed to investigate the relationship between Fe absorption, Fe requirements and standard haematological indices in IBD patients without active disease. Additionally, it assessed the formation of circulating NTBI in patients with IBD and controls following ingestion of ferrous sulphate.

## Experimental methods

### Participants

Patients with IBD ( $n$  29: five with ulcerative colitis and twenty-four with Crohn's disease) were recruited from gastrointestinal outpatient clinics at Guy's and St Thomas' NHS Hospital Trust, London, UK. Control subjects ( $n$  28) were recruited from a local newspaper advert.

### Patients

Patients were aged 18–65 years and in all cases, IBD was diagnosed by histological and/or radiological criteria. Patients with other chronic diseases, hereditary disorders of Fe metabolism (detected by the assessment of common mutations in the *HFE* gene using 'WAVE<sup>®</sup>' technology based on denaturing HPLC), pregnant and lactating females and those taking proton-pump inhibitors or Fe therapy/supplements within the previous 28 d were excluded. Additionally, only patients with inactive or mildly active disease using a Harvey–Bradshaw index (HBI) of less than 8<sup>(18)</sup> were recruited for the study. Patients fulfilling these criteria were invited to participate and then had a blood sample taken to assess Fe status (full blood count, ferritin, serum Fe, soluble transferrin receptor and total serum Fe-binding capacity (TIBC)) and inflammatory status (erythrocyte sedimentation rate and C-reactive protein).

### Controls

An advert was placed in a freely available newspaper distributed predominantly within Greater London. Potential subjects

responding to the advert were screened by telephone to exclude anyone with known chronic disease, gastrointestinal disorders, hereditary disorders of Fe metabolism and those taking proton-pump inhibitor medication or Fe therapy/supplements within the previous 28 d. Pregnant and lactating women were also excluded. Volunteers fulfilling these criteria were invited to participate and then had a blood sample taken to assess Fe status and inflammatory status, as detailed above. Mutations of the *HFE* gene were also assessed.

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the St Thomas' Hospital Local Research Ethics Committee (EC03/089). Written informed consent was obtained from all subjects.

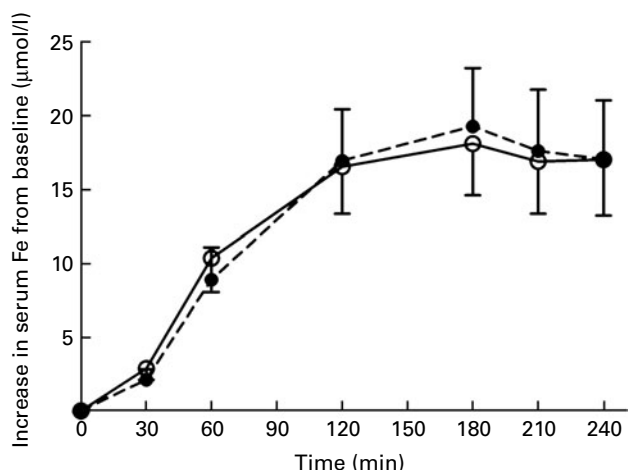
### Study design

Recruited subjects were invited to attend St Thomas' Hospital, London (UK) for a single 4 h study appointment at a mutually agreeable time. Subjects were requested not to take any multivitamin or mineral supplements for the week preceding the study and were advised to avoid any Fe-rich foods and tea on the day of their appointment. Subjects were not fasted as it has previously been shown that Fe absorption from oral ferrous sulphate is similar with or without fasting<sup>(10)</sup>.

Upon arrival, signed consent was obtained and each subject was cannulated in the forearm with a 21G Venflon Pro cannula (Becton Dickinson Infusion Therapy AB, Helsingborg, Sweden) and a 20 ml baseline blood sample was taken using a plastic 20 ml syringe. The collected blood was divided into aliquots into labelled plain, EDTA and SST<sup>®</sup> (serum separating tube) vacutainers (Becton Dickinson Vacutainer Systems, Plymouth, UK) for the baseline measures as outlined above. Following collection of the baseline blood sample, subjects were given a single ferrous sulphate capsule (200 mg; 65 mg elemental Fe) with a glass of water. Further blood samples (10 ml) were collected at 30, 60, 120, 180, 210 and 240 min post-Fe ingestion to measure serum Fe, TIBC and NTBI.

### Laboratory analysis

Baseline samples for ferritin and C-reactive protein (SST vacutainer) and full blood count (EDTA vacutainer) were analysed by the Clinical Chemistry Laboratory using routine laboratory methods. Samples collected in plain vacutainers for serum Fe, NTBI and TIBC were immediately taken following collection (on ice) to a laboratory where the samples were allowed to clot before being centrifuged at 2500 rpm for 10 min at 4°C (Eppendorf 5804R; Eppendorf, Hamburg, Germany). Following centrifugation, serum was transferred to labelled cryovials, frozen and stored at –80°C in labelled boxes. Samples for serum Fe, TIBC and serum transferrin receptor analysis were analysed by routine methods at the Nutritional Biochemistry Laboratory, MRC Human Nutrition Research, Cambridge. Serum NTBI was analysed using a modified version of the method developed by Singh *et al.*<sup>(19)</sup> in the Department of Pharmacy, King's College London, as described previously<sup>(20)</sup>.



**Fig. 1.** Increase in serum iron from baseline following ferrous sulphate administration in patients with inflammatory bowel disease ( $n$  29;  $\circ$ ) *v.* control subjects ( $n$  28;  $\bullet$ )  $P=0.9$ . Values are means, with standard errors of the mean represented by vertical bars.

Transferrin saturation was calculated from TIBC using the following equation:

$$\text{Transferrin saturation} = 100 \times (\text{serum Fe}/\text{TIBC}).$$

#### Data and statistical analysis

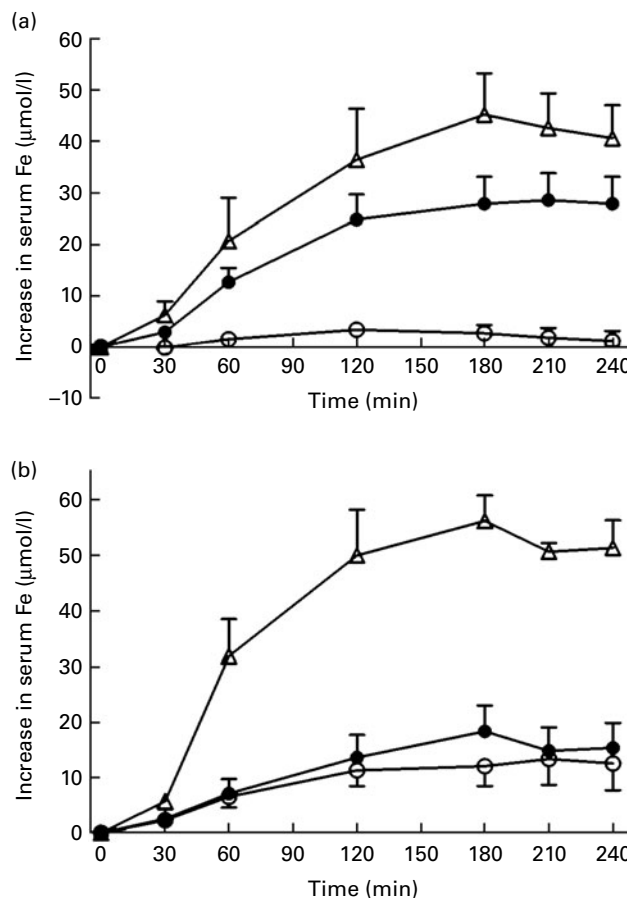
Subjects with non-Fe-deficiency anaemia, significant inflammation or homozygous mutations of the *HFE* gene were excluded from the study. Subjects where venous access could not be maintained for the duration of the study were excluded from the analysis. Data were analysed using SPSS version 14 (SPSS, Inc., Chicago, IL, USA) and are presented as means and standard deviations or, where indicated, as means with their standard errors. Unpaired *t* tests were used to make between-group comparisons of the peak serum Fe and comparison of baseline markers, of Fe status and inflammation, between control subjects and patients with IBD. Pearson's product moment correlation coefficient was used to measure the correlation between NTBI, serum Fe and transferrin saturation. Significance was assumed where  $P < 0.05$ .

#### Results

A total of seventy-two subjects (IBD  $n$  36, controls  $n$  36) were screened for study recruitment. Of these, five subjects in the control group were not suitable due to non-Fe-related anaemia and two patients with IBD were excluded, one due to moderately active disease (HBI = 12) and the other because he was homozygous for the primary haemochromatosis-susceptibility mutation, C282Y. The remaining sixty-five subjects consented to take part but data were incomplete for eight (IBD  $n$  5, controls  $n$  3) due to difficulty in maintaining venous access for the duration of the study and were excluded from the analysis. Of the remaining fifty-seven subjects, twenty-eight subjects were controls (twelve males), with a mean age of 35 (SD 11) years and BMI of 23.4 (SD 3) kg/m<sup>2</sup>,

and twenty-nine had IBD (thirteen males), with a mean age of 42 (SD 13) years, BMI of 25.7 (SD 6) kg/m<sup>2</sup> and HBI of 4.2 (SD 1.8). Of these patients with IBD, five had ulcerative colitis and the remaining patients had Crohn's disease (site involved: ileal  $n$  4; ileocaecal  $n$  3; ileocolonic  $n$  8; colonic  $n$  5; site not specified  $n$  4).

Fe absorption was measured by the rise in serum Fe over 4 h, following ingestion of ferrous sulphate, and was similar for patients with IBD ( $n$  29) and control subjects ( $n$  28) (Fig. 1). According to the *a priori* classification of Fe status (see the Methods section), control subjects with ID ( $n$  10) or IDA ( $n$  5) had peak Fe absorption about 180 min post-dose and approximately 30 and 45  $\mu\text{mol/l}$  above baseline levels, respectively. In contrast, control subjects without ID (i.e. Fe replete;  $n$  13) had Fe absorption peaks that increased by less than 5  $\mu\text{mol/l}$  (control subjects: IDA or ID *v.* IR  $P < 0.001$ ; Fig. 2). In patients with IBD and IDA ( $n$  4), a significant increase in serum Fe levels, peaking at 55  $\mu\text{mol/l}$  above baseline, was observed while in non-anaemic patients with IBD with ID ( $n$  11) or without ID



**Fig. 2.** Increase in serum iron from baseline following ferrous sulphate administration in (a) control subjects ( $n$  13 iron repletion (IR)  $\circ$ ,  $n$  10 iron deficiency (ID)  $\bullet$  and  $n$  5 iron-deficiency anaemia (IDA)  $\triangle$ ) and (b) patients with inflammatory bowel disease (IBD) ( $n$  14 IR  $\circ$ ,  $n$  11 ID  $\bullet$  and  $n$  4 IDA  $\triangle$ ) according to the *a priori* classification of iron status. For patients with IBD,  $P=0.8$  for IR *v.* ID and  $P < 0.05$  for IR *v.* IDA. For control subjects,  $P < 0.001$  for IR *v.* ID or IDA. Values are means, with standard errors of the mean represented by vertical bars. All according to standard haematological criteria (see the Methods section).

(i.e. IR; *n* 14), similar Fe absorption curves were observed with mean peak increases of approximately 15  $\mu\text{mol/l}$  (patients with IBD: ID *v.* IR *P*=0.8; Fig. 2).

Due to the inability of Fe status markers to predict Fe absorption, in the absence of anaemia in subjects with IBD (Fig. 2), further assessment was undertaken. The peak rise in serum Fe is a strong correlate for total Fe absorption<sup>(9)</sup>. Subjects were thus categorised according to whether they did or did not absorb Fe, and this was related to baseline ferritin, serum Fe and transferrin saturation in all subjects. Briefly, the Fe status of all subjects was determined using reference ranges for haematological values, so subjects with ID were classified as having (1) serum ferritin <20  $\mu\text{g/l}$  or (2) serum ferritin 20–55  $\mu\text{g/l}$  and either serum Fe <14  $\mu\text{mol/l}$  or transferrin saturation <10%<sup>(21)</sup>. Mild anaemia was defined as Hb concentration ranging from 110 to 129 g/l for males and 105 to 124 g/l for females. Additionally, subjects were also categorised as Fe absorbers or non-absorbers, defined as having a peak serum Fe increase of more than 5  $\mu\text{mol/l}$  or less than 5  $\mu\text{mol/l}$ , respectively, following ingestion of 65 mg Fe as ferrous sulphate. In the control subjects, all four markers (baseline ferritin, serum Fe, transferrin saturation and Hb) provided some significant prediction of Fe requirements (i.e. absorption) as expected, whereas there was no significant predictive power of these markers in patients with IBD (Table 1).

Total Fe absorption, transferrin saturation and NTBI were all strongly correlated for both subject groups (Fig. 3). Surprisingly, even baseline NTBI (i.e. pre-Fe dose) was present in some individuals and, again, correlated with

transferrin saturation (Fig. 3). NTBI in the absence of transferrin saturation with Fe is controversial and, therefore, these data suggest its presence as either an artifact of NTBI assays or an equilibrium product with Fe transferrin, regardless of whether samples come from subjects who are healthy or with IBD. This is further discussed below.

### Discussion

The present findings provide important information on a number of aspects of Fe status and Fe absorption in subjects with IBD.

First, in spite of speculation, Fe absorption is normal in patients with IBD without significant inflammation when compared as a group with healthy controls, and as opposed to patients with active IBD<sup>(5)</sup>. We recognise that the HBI is used for assessing clinical activity (i.e. a proxy for inflammation) in patients with Crohn's disease and not with ulcerative colitis, but only five out of twenty-nine had ulcerative colitis in the present study and the findings were the same with all IBD patients (*n* 29) or just Crohn's disease patients (*n* 24) (data not shown).

Second, IDA in patients with IBD, when assessed by traditional haematological criteria, is predictive of high Fe needs (Fig. 2), whereas ID alone is not (i.e. in the absence of concomitant anaemia) and Fe repletion is not predictive of low Fe needs (Fig. 2). Thus, while patients in this cohort had quiescent or mild IBD and only slight increases in systemic inflammatory markers such as C-reactive protein, erythrocyte sedimentation rate and

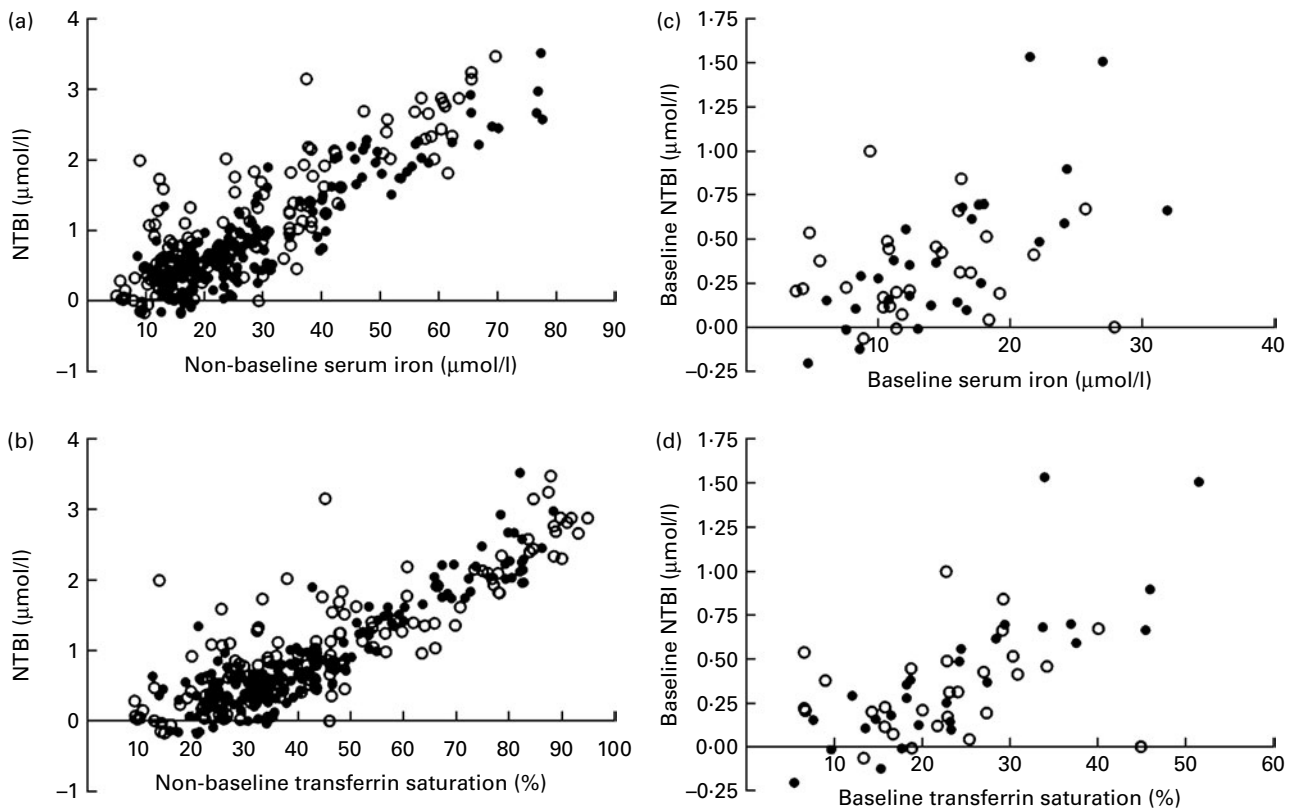
**Table 1.** Comparison of baseline markers of iron status (and inflammation) in control subjects and patients with inflammatory bowel disease (IBD) classified as iron absorbers and non-absorbers\*

(Mean values and standard deviations, medians and range values)

	Control absorbers ( <i>n</i> 18)				Control non-absorbers ( <i>n</i> 10)				<i>P</i>
	Mean	SD	Median	Range	Mean	SD	Median	Range	
Hb (g/l)	129	12	131	104–152	148	20	146	130–195	0.005
Ferritin ( $\mu\text{g/l}$ )	25	22	21	3.0–90.0	99	67	105	15–203	<0.001
Serum transferrin receptor (mg/l)	4.7	1.9	4.2	3.1–11.0	3.9	0.4	3.9	3.2–4.7	0.2
Serum Fe ( $\mu\text{mol/l}$ )	12.6	5.1	11.8	4.7–22.2	20.9	7.1	18.7	11.5–33.0	0.004
TIBC ( $\mu\text{mol/l}$ )	71	10	72	54.8–91.5	57	8	53	48.6–70.2	<0.001
NTBI ( $\mu\text{mol/l}$ )	0.3	0.4	0.2	–0.2–1.5	0.7	0.3	0.6	0.4–1.5	0.005
Transferrin saturation (%)	23.1	8.2	22.0	10.0–38.0	34.0	11.8	36.0	21–50	<0.001
ESR (mm/h)	5	4	3	1.0–13.0	2	1	2	2–3.0	0.1
CRP (mg/l)	5.1	0.3	5.0	5.0–6.0	5.8	1.6	5.0	5.0–9.0	0.07
Platelets ( $10^9$ )	234	45	232	141–321	216	34	220	168–278	0.3
	IBD absorbers ( <i>n</i> 21)				IBD non-absorbers ( <i>n</i> 8)				
	Mean	SD	Median	Range	Mean	SD	Median	Range	<i>P</i>
Hb (g/l)	133	15	133	88–160	140	8	140	130–149	0.3
Ferritin ( $\mu\text{g/l}$ )	39	23	39	5.0–94.0	39	14	38	22–62	0.7
Serum transferrin receptor (mg/l)	4.4	1.2	4.3	2.6–7.5	4.1	1.0	4.4	2.0–4.9	0.7
Serum Fe ( $\mu\text{mol/l}$ )	16.3	7.7	15.7	3.0–30.2	13.9	7.2	13.2	5.6–27.9	0.5
TIBC ( $\mu\text{mol/l}$ )	61	10	62	41.0–80.0	57	10	59	42.1–72.5	0.3
NTBI ( $\mu\text{mol/l}$ )	0.4	0.3	0.3	0.0–1.0	0.2	0.3	0.2	–0.1–2.7	0.3
Transferrin saturation (%)	21.3	8.5	22.8	6.6–40.1	30.4	16.9	30.0	7.0–63.0	0.2
ESR (mm/h)	14	11	9	5.0–42.0	10	9	7	2.0–26.0	0.4
CRP (mg/l)	8.4	7.7	6.0	5.0–39.0	10.7	8.6	7.0	5.0–28.0	0.5
Platelets ( $10^9$ )	293	62	290	199–457	276	64	274	170–381	0.5

TIBC, total Fe-binding capacity; NTBI, non-transferrin-bound Fe; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

\* See the Methods section for the categorisation of Fe absorbers and non-absorbers.



**Fig. 3.** Correlation between non-transferrin-bound iron (NTBI) and (a) non-baseline serum iron ( $r^2$  0.74;  $P < 0.001$ ), (b) non-baseline transferrin saturation ( $r^2$  0.77;  $P < 0.001$ ), (c) baseline serum iron ( $r^2$  0.24;  $P < 0.001$ ) and (d) baseline transferrin saturation ( $r^2$  0.40;  $P < 0.001$ ) in patients with inflammatory bowel disease (IBD;  $n$  29; ○) and control subjects ( $n$  28; ●).  $r^2$  and  $P$  values are for patients with IBD and control subjects combined. Non-baseline refers to all values that were not at baseline (i.e. all time points post-iron dose).

platelets, this appears sufficient to affect measures based on Fe, transferrin and ferritin as markers of Fe requirements as is well known with more severe inflammation. Levels of serum soluble transferrin receptor have been proposed to be less influenced by inflammation than levels of circulating ferritin or Fe/transferrin<sup>(22)</sup>, but this measure was also insensitive in assessing Fe needs in IBD. Thus, in the absence of anaemia, current haematological parameters are unable to predict Fe stores, and hence Fe requirements, even in patients with quiescent or mildly active IBD. Given that oral ferrous Fe supplementation has, on occasions, been associated with severe gastrointestinal adverse effects in IBD<sup>(23)</sup>, as also demonstrated in animal models of colitis<sup>(24–26)</sup>, these data suggest that Fe supplementation should only be undertaken in subjects with ID and IDA where Fe needs are clear. Even then, anaemia of chronic disease, which normally is associated with active IBD, should be ruled out because Fe absorption is then markedly down-regulated<sup>(5)</sup>. Diagnostic criteria for the assessment of ID and IDA in IBD in the presence and absence of inflammation have been developed<sup>(27)</sup>.

Third, in agreement with previous work<sup>(10,11)</sup>, the present study has shown that following a single dose of oral ferrous sulphate, there is a rise in apparently detectable NTBI in the circulation of control subjects and in patients with IBD (Figs. 1 and 3). Transferrin is not just an Fe transport protein but is also an anti-redox substrate, so any circulating Fe that

is not bound to transferrin (i.e. NTBI) has the capacity for systemic oxidative activity. Individuals with low mucosal and systemic antioxidant status, as has been proposed for patients with IBD<sup>(15–17)</sup>, could, in theory, be especially prone to NTBI effects, including vascular damage and lipid peroxidation<sup>(13,14,28)</sup>. Unsurprisingly, the detection of NTBI in serum was strongly correlated with total Fe uptake into serum and, therefore, the degree of transferrin saturation (Fig. 3). However, unexpectedly, baseline serum Fe levels and transferrin saturation (i.e. fasting and pre-ferrous sulphate) also correlated with NTBI (Fig. 3). This raises the question of whether, outside of the Fe overload, NTBI genuinely exists as a function of partially saturated transferrin or is an artifact of NTBI assays (i.e. do NTBI assays compete for a small fraction of transferrin-bound Fe in proportion to total serum Fe concentration?). Although beyond the scope of the present study, these issues should be resolved in further work, as it is important to establish whether circulating NTBI really occurs following supplemental oral Fe.

In summary, (1) there was no evidence for Fe absorption to be dysfunctional in patients with IBD. (2) In quiescent or mildly active IBD, IDA clearly indicates a need for Fe repletion. (3) In the absence of anaemia, standard haematological criteria cannot predict Fe requirements in IBD. (4) A signature for NTBI is detected in patients with IBD to the same extent as it is detected in control subjects, following ingestion



of ferrous sulphate, but further work is required to determine the authenticity of this apparent NTBI.

Overall, the implications of the present study are that if IBD patients with ID are to be supplemented with Fe, then they should have a concomitant anaemia consistent with IDA (typically a microcytic anaemia). Based on previous work, optimal Fe absorption (i.e. supplemental effectiveness) will be inhibited in patients with active disease<sup>(5)</sup>, while oral Fe may still exacerbate symptoms<sup>(23)</sup> so the choice between enteral and parenteral Fe supplementation, and which patients to target, should be carefully considered.

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