Original Article

Title: Quantitative data on the magnitude of the systemic inflammatory response and its relationship with serum measures of iron status.

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Abbreviations

AGP α-1-acid glycoprotein

CRP C-reactive protein

F female

IL interleukin

LOD limit of detection

M male

MCH mean corpuscular hemoglobin

MCV mean corpuscular volume

sTFR soluble transferrin receptor

TIBC total iron binding capacity

TSAT transferrin saturation,

Abstract

The present study aimed to quantify the magnitude of the systemic inflammatory response, measured by C-reactive protein (CRP) and albumin, and its relationship with common serum biochemical measures of iron status including total iron, transferrin, transferrin saturation and ferritin. Retrospective interrogation of laboratory computer databases at 4 centres between 2006 and 2011 provided results from patients in which serum CRP and albumin had been measured together with iron studies (iron, transferrin and transferrin saturation, n=16,522) and ferritin (n=7,226). Analyte results were categorized into groups according to CRP and albumin. When those groups with CRP <10mg/L and albumin >35g/L, CRP11-80mg/L and albumin 25-35mg/L, and CRP >80mg/L and albumin <25g/L were compared, the median serum total iron was 15.0, 7.0 and 3.0 μmol/L respectively ($p<0.001$), an overall reduction of 80%. The median serum transferrin concentration was 2.6, 2.0 and 1.3 μmol/L respectively ($p<0.001$), an overall reduction of 50%. The median transferrin saturation was 23%, 13% and 10% respectively ($p<0.001$), an overall reduction of 56%. The median serum ferritin was 77, 173 and 445 μg/L respectively ($p<0.001$), an overall increase of 578%. The present study quantifies the impact of the systemic inflammatory response on serum measures of iron status. This association should be taken into account when measures of iron status are requested and interpreted to prevent misdiagnosis.

Introduction

The gold standard assessment of iron status is considered to be microscopy of a bone marrow trephine sample, however, this is a painful and invasive procedure.[1] There are a number of serum analytes proposed for the assessment of iron status. These include iron itself, proteins involved in its metabolism such as transferrin, ferritin, soluble transferrin receptor (sTFR) and zinc protoporphyrin, and derived values such as transferrin saturation (TSAT) and total iron binding capacity (TIBC).[2]

There is evidence that the presence of systemic inflammation is associated with decreased serum concentrations of iron and transferrin, which are negative acute phase reactants.[3] In contrast, ferritin is a positive acute phase protein and its serum concentrations rise in the presence of systemic inflammation.[4] Indeed, current WHO guidance on assessment, and interventions in iron status recommend that the presence of inflammation be considered when ferritin levels are measured in those who are apparently well.[5] However, the magnitude of the impact is not well quantified in patients.

There is increasing evidence that the presence of a systemic inflammatory response confounds the interpretation of a number of serum micronutrients.[6] Indeed, recent studies have quantified the impact of the systemic inflammatory response, as evidenced by both serum C-reactive protein (CRP) and albumin, on serum vitamins and micronutrients.[7-8] With reference to iron status, hepcidin is a key regulator of iron homeostasis, acting to reduce iron export and cause sequestration of iron through the inhibition of ferroportin.[9] It is thought that hepcidin synthesis is influenced by cytokines such as interleukin 6 (IL 6), which promotes iron uptake by cells of the innate immune system.[10] Similar to hepcidin, the synthesis of CRP by hepatocytes is

driven by circulating IL 6.[11] In addition, albumin, although it is not directly involved in iron transport, is quantitatively the most important circulating binding protein, and is itself a negative acute phase protein commonly measured in clinical practice.[12]

Therefore the aim of the present study was to quantify the impact of the magnitude of systemic inflammation, as evidenced by CRP and albumin, on commonly measured serum biochemical measures of iron status. Furthermore the study was carried out with the aim of developing local guidelines for the interpretation of serum measurements of iron status.

Materials and Methods

Patients:

Details of all requests for iron studies (total iron, transferrin, and derived transferrin saturation) and ferritin were obtained from the biochemistry and haematology laboratory information systems of 4 Glasgow hospitals respectively for the period 1st August 2006 to 31st July 2011. These requests were made from secondary inpatient, secondary outpatient and primary care healthcare providers. Iron study and ferritin results were matched to CRP and albumin results obtained on the same calendar day using electronic laboratory patient identifiers. Any iron study requests which were not accompanied by a CRP and albumin request were not included in the study. Where an individual had repeat measurements, only the first was included in the study.

Two groups were obtained: a large cohort of patients who had iron study measurement, including total iron and transferrin results (n=16,522), and a smaller cohort of patients who had ferritin results (n=7,226). The dataset of ferritin results was not a subset of the larger iron study dataset.

The audit was conducted with the intent of developing local guidelines and to aid in the interpretation of serum measurements of iron status and was approved by NHS Greater Glasgow and Clyde .

Methods:

Serum total iron (chemically using ferene), transferrin (by the immunoturbidimetric method), CRP (by the immunoturbidimetric method) and albumin (chemically using Bromocresol purple) were measured using an automated analyser (Architect, Abbot

Diagnosis, Maidenhead, UK) in the routine biochemistry laboratories. Ferritin was analysed using 2 step chemiluminescent microparticle immunoassay within the routine haematology laboratories. All sites used the same analytic materials and automated platforms. There were no sustained concerns regarding IQC performance requiring investigation into the performance of the assays. The A, B and C scores were within the EQA (NEQAS) targets during the study period. Transferrin saturation was calculated empirically from serum total iron and transferrin by each laboratory as (4 * [Fe] nmol/L) / (Transferrin mg/L) assuming 2 iron molecules bind to one transferrin molecule. Although the theoretical problems with this method are noted, this was the calculation used in all laboratories during the study period.[13] Where a calculated transferrin saturation result was not available as either the iron or transferrin results were below the limit of detection (LOD, <2μmol/L and <0.2g/L respectively), the results below the LOD for transferrin saturation was estimated by substituting the absolute value of the LOD.

Statistical analysis:

Data was presented as median and ranges. Correlations between variables were carried out using Spearman's rank method (r_s). The cohorts were divided into groups according to CRP (<10, 10-80, >80mg/L) and albumin concentrations (<25, 25-35, >35 g/L) as previously described.[8, 14-15] Furthermore, from these initial groupings, 3 groups with minimal (group 1, CRP <10mg/L and albumin >35g/L), moderate (group 2, CRP 10-80mg/L and albumin 25-35g/L) and maximal (group 3 CRP >80mg/L and albumin <25g/L) systemic inflammation were identified. The distribution of each

parameter of iron status were compared across strata of CRP and albumin using the Kruskal-Wallis test.

The proportion of individuals with results outside the laboratory reference ranges of serum total iron (10-30 μmol/L) and transferrin (2.0-4.0 g/L) was calculated and compared using the χ^2 test for linear association. The proportion of individuals in each stratum meeting common criteria for iron deficiency or excess as defined by serum transferrin saturation and ferritin was calculated and compared using the χ^2 test for linear association. Cut off values for iron deficiency normally applied to patients without inflammation were; transferrin saturation <10%, and ferritin <15μg/L.[16] Cut off values for iron excess normally applied to patients without inflammation were; transferrin saturation >55% in males or >50% in females, ferritin >300μg/L in males or >200μg/L in females.[17]

P values <0.05 were considered statistically significant. Statistical analyses were performed using IBM SPSS version 22 for Windows (Chicago, IL, USA).

Results

Patients:

During the study period there were 16,522 patients (F/M: 56%/44%; median age: 68; interquartile range: 51 to 79 years) in the iron study cohort (iron, transferrin, calculated transferrin saturation), and7,226patients (F/M: 55%/45%; median age: 68; interquartile range: 52 to 79 years) in the ferritin cohort (Table 1). There was no significant difference in age (p=1.000), sex (p=0.309), median CRP (14.0mg/L vs. 15mg/L, p=0.248) or median albumin (33g/L vs. 32 g/L, p=0.980) when the two cohorts were compared. It did not therefore influence further the way in which the data was analysed.

All iron status measures were significantly associated with CRP, and albumin (Table 2). There was a significant inverse correlation between age and iron (r_s=-0.301, p<0.001)), transferrin (r_s=-0.161, p<0.001), transferrin saturation (r_s=-0.237, p<0.001), and ferritin (r_s=-0.023, p=0.047) for the sexes combined. When the sexes were compared, there was a small but statistically significant difference in median iron (M=9.0µmol/L, F=8.0µmol/L, p<0.001), transferrin (M=2.00g/L, F=2.20g/L, p<0.001), transferrin saturation (M=19%, F=15%, p<0.001), and ferritin (M=233µg/L, F=115µg/L).

Serum Total Iron:

Serum total iron was significantly inversely correlated with CRP (r_s=-0.554, p<0.001) and positively correlated with albumin (r_s=0.907, p<0.001). When systemic inflammation groups 1 (CRP <10mg/L and albumin >35g/L), 2 (CRP 11-80mg/L and albumin 25-35mg/L), and 3 (CRP >80mg/L and albumin <25g/L) were compared (Table 3), the median serum total iron was 15.0, 7.0 and 3.0 µmol/L respectively

(p<0.001) with group 3 having a median serum total iron 80% lower than that of group 1. When systemic inflammation groups 1, 2 and 3 were compared, there was a significant difference in the proportion of patients with serum total iron below (<10 μmol/L, 29%, 76% and 93% respectively, p<0.001) or above (>30 μmol/L, 8%, 2% and 1% respectively, p<0.001) the reference range.

Transferrin:

Serum transferrin was significantly inversely correlated with CRP (r_s=-0.511, p<0.001) and positively correlated with albumin (r_s=0.679, p<0.001). When systemic inflammation groups 1 (CRP <10mg/L and albumin >35g/L), 2 (CRP 11-80mg/L and albumin 25-35mg/L), and 2 (CRP >80mg/L and albumin <25g/L) were compared (Table 4), the median serum transferrin concentration was 2.6, 2.0 and 1.3 g/L respectively (p<0.001) with group 3 having a median serum transferrin 50% lower than that of group 1. When systemic inflammation groups 1, 2 and 3 were compared, there was a significant difference in the proportion of patients with serum transferrin below (<2.0 g/L, 12%, 54% and 96% respectively, p<0.001) or above (>4.0 g/L, 2%, 0% and 0% respectively, p<0.001) the reference range.

Transferrin saturation:

Transferrin saturation was significantly inversely correlated with CRP (r_s=-0.349, p<0.001) and positively correlated with albumin (r_s=0.161, p<0.001). When systemic inflammation groups 1 (CRP <10mg/L and albumin >35g/L), 2 (CRP 11-80mg/L and albumin 25-35mg/L), and 3 (CRP >80mg/L and albumin <25g/L) were compared (Table 5), the median transferrin saturation was 23%, 13% and 10% respectively (p<0.001) with group 3 having a median transferrin saturation 56% lower than that of

group 1. When systemic inflammation groups 1, 2 and 3 were compared, there was a significant difference in the proportion of patients meeting criteria for iron deficiency (TSAT <10%, 15%, 39% and 53% respectively, p<0.001) or iron excess (TSAT M>55% F>50%, 7%, 5% and 5% respectively, p<0.001).

Ferritin:

Serum ferritin was significantly positively correlated with CRP (r_s=0.396, p<0.001) and inversely correlated with albumin (r_s=-0.383, p<0.001). When systemic inflammation groups 1 (CRP <10mg/L and albumin >35g/L), 2 (CRP 11-80mg/L and albumin 25-35mg/L), and 2 (CRP >80mg/L and albumin <25g/L) were compared (Table 6), the median serum ferritin was 77, 173 and 445 μg/L respectively (p<0.001) with group 3 having a median serum ferritin 578% higher than that of group 1. When systemic inflammation groups 1, 2 and 3 were compared, there was a significant difference in the proportion of patients meeting criteria for iron deficiency (<15 μg/L, 13%, 3% and 0% respectively, p=0.001) or iron excess (M>300 μg/L F>50 μg/L, 21%, 38% and 75% respectively, p<0.001).

Discussion

The results of the present study show that serum measures of iron status, iron, transferrin, transferrin saturation and ferritin are significantly and independently associated with CRP and albumin. Serum total iron, transferrin and ferritin concentrations were particularly sensitive to CRP and albumin concentrations outside the normal ranges, such that there was a synergistic impact on these measures of iron status. A clear conclusion is that determining the iron status of patients with acute or ongoing systemic inflammation using these serum measures is problematic.

Indeed, according to the results of the present study, many patients with CRP and albumin concentrations outside the normal range would meet diagnostic criteria for iron deficiency by iron and transferrin saturation but would also meet criteria for iron overload as measured by serum ferritin.[5] The clinical implications are profound, for example based on such serum measures there may be inappropriate use of iron replacement therapy or blood transfusion in those with systemic inflammation during medical or surgical care. Therefore, given external validation of these findings, the possibility of the presence of systemic inflammation should be considered in all patients for whom iron status tests are requested to allow health care practitioners to appropriately diagnose iron deficiency or excess.

It is also of interest that some studies have reported a significant association between serum measures of iron status such as transferrin saturation and ferritin, and increased mortality from any cause, cancer and cardiovascular disease.[18-19] Given that CRP and albumin also predict mortality from any cause, cancer, cardiovascular disease and cerebrovascular causes, it may be that the association of measures of iron status and mortality is dependent on the presence of systemic inflammation.[20] Further work is required to test this hypothesis.

In the present study, serum CRP and albumin were used as they are routine, clinically available measures of the magnitude of systemic inflammation, however there are other markers of inflammation that are used in clinical practice. Where there is a chronic inflammatory state, iron status can be determined using indices that directly describe the erythrocyte population, such as hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), or peripheral blood film microscopy.[21] It is well understood that erythropoiesis is affected by both chronic and acute inflammation however the above indices are less subject to rapid change than acute phase proteins and are less likely to be useful in acute systemic inflammation.[9] Platelets are also routinely measured as part of a full blood count (FBC). Indeed, thrombocytosis is well recognised to be associated with both systemic inflammation and iron deficiency.[22-23] Components of the differential white cell count including a variety of ratios derived from neutrophils, monocytes and lymphocytes have been of interest, particularly in the setting of clinical oncology.[24-25] However relationships between these ratios, such as the neutrophil lymphocyte ratio (NLR) and lymphocyte monocyte ratio (LMR) and iron status are unclear.

Serum procalcitonin concentrations have been used in clinical practice to identify the presence of bacterial infection in particular, especially in the critical care setting, however to our knowledge the relationship between procalcitonin and measures of iron status has not been examined.[26] Theses markers of the systemic inflammation response do not readily differentiate the source of inflammation and their interpretation is dependent on the clinical context. There is also increasing interest in the use of soluble transferrin receptor (sTFR) in the assessment of iron status as it is less affected by systemic inflammation.[27] The use of the sTFR/log

ferritin index has also been proposed.[28] However, at present sTFR is not widely available in UK clinical practice, and is subject to substantial biological variability.[29]

A potential limitation of the present study is the lack of clinical data regarding reasons for the investigation of iron status in the included patients. In particular, other than that of albumin, there were no clinical data relating to the presence of liver disease or markers of synthetic liver function which is associated with altered iron homeostasis. However, given the large number of patients in the cohorts analysed in the present study it would be unlikely that the associations identified were due to the sampling of a specific population.. The consistent trends of progressive changes in analyte concentration with differing CRP and albumin provide convincing evidence of the association between inflammation and these measures. Moreover, these observations can be readily externally validated.

The present study demonstrates a significant association between commonly measured serum biochemical measures of iron status and the magnitude of systemic inflammation as measured by serum CRP and albumin. This association should be taken into account when measures of iron status are requested and interpreted to avoid misdiagnosis. However, given the relative variability of the association between the systemic inflammatory response and these serum measures of iron status, attempts to mathematically correct for it using regression equations for CRP and albumin are likely to prove relatively inaccurate. It may be that in future, assays less influenced by systemic inflammation, e.g. soluble transferrin receptors, will be routinely available to reliably diagnose states of iron deficiency or excess in patients referred for iron status assessment. Furthermore, if there is evidence of systemic

inflammation, the serum measure of iron status assessed in the present study should not be used to routinely assess iron status.

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Tables and footnotes:

Table 1: Characteristics of patients in iron studies and ferritin cohorts

Table 2: Correlation between serum iron status measures, C-reactive protein and albumin

Table 3: Distribution of serum Iron and proportion of patients below (<10 μmol/L) and above (>30 μmol/L) the reference range according to C-reactive protein and albumin concentrations

Table 4: Distribution of serum Transferrin and proportion of patients below (<2.0 g/L) and above (>4.0 g/L) the reference range according to C-reactive protein and albumin concentrations

Table 5: Distribution of serum Transferrin saturation and proportion of patients meeting criteria for iron deficiency (<10%) and excess (male >55%, female >50%) according to C-reactive protein and albumin

Table 6: Distribution of serum Ferritin and proportion of patients meeting criteria for iron deficiency (<15 μg/L) and excess (male >300μg/L, female >200 μg/L) according to C-reactive protein and albumin