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## **Additional background**

Subjects homozygous for C282Y detected by genetic testing will often show abnormalities in iron metabolism (raised transferrin saturation and serum ferritin) but heterozygotes show only minor changes and rarely accumulate significant iron overload.<sup>1</sup> One Australian survey has shown serum ferritin to be related to meat intake in a healthy population,<sup>2</sup> but to date no genetic studies have considered the influence of dietary iron intake in relation to these mutations and levels of iron storage. Regular phlebotomy is a simple and acceptable treatment for homozygotes, but the potential for additional dietary regulation is not known, or whether dietary intake is as relevant to the larger group of heterozygotes as to homozygotes.

## **Additional methods**

### DNA extraction

DNA extraction from cheek cells was carried out using a rapid alkaline lysis method similar to that of Rudbeck and Dissing.<sup>3</sup> This was coupled with a highly sensitive fluorescent ARMS (Amplification Refractory Mutation System) technique.<sup>4,5</sup> All women found to be heterozygote or homozygote for C282Y were re-contacted to provide a blood sample for confirmation of genotype and measurement of iron status. Women whose genotype could not be determined with accuracy from their cheek cells were also asked to provide a blood sample.

### Analysis of serum ferritin concentration

All blood samples were analyzed for serum ferritin concentrations (storage iron) by a two-site chemiluminometric (sandwich) immunoassay (Bayer, Newbury, UK) and for serum iron and transferrin saturation (transport iron). Serum iron and unbound iron binding capacity (UIBC) were determined on a Hitachi automated analyzer (Roche, Lewes, UK). Total iron binding capacity (TIBC) was calculated (serum iron + UIBC) and transferrin saturation ( $\text{serum iron} / \text{TIBC} \times 100\%$ ). Hemoglobin levels were measured as an assessment of functional iron status using a Coulter STKR analyzer (Coulter Beckman, High Wycombe, UK).

### Dietary analysis

The nutrient content of each of the 217 items in the food frequency questionnaire (FFQ), including total iron intake, were derived from standard UK Food Composition tables.<sup>6</sup> In the past, heme iron content of meat has often been taken to be 40% of the total iron it contains<sup>7-9</sup> but in practice the proportion depends upon the type of meat consumed. Based on an extensive literature search, the heme iron content of each type of meat, fish and poultry was estimated and applied to the meat items in the FFQ<sup>10</sup>. Using this approach, 100g of roast beef was estimated to contain 1.28 mg of heme iron, for example, whilst roast chicken was estimated to contain 0.22 mg/100g. Supplemental intake of iron and other minerals and micronutrients was assessed by separate questionnaire.

### Power calculation

Power to detect the gene-diet interaction was estimated using the method of Luan et al.<sup>11</sup> and confirmed by substantial computational simulations and sensitivity analyses. The study had 90% power to detect a regression slope for homozygotes three times higher than heterozygotes and wild type combined (based on standardized heme iron intake and standardized, log-transformed serum ferritin concentrations). This would also give 90% power to detect a ratio of 1.5 for the regression slopes for heterozygotes compared to wild type.

### **Unadjusted results**

Before adjusting for potential confounders, serum ferritin was 2.9 times higher (99% CI = 2.1 - 4.2) in C282Y homozygotes than wild types. Serum iron was 14 (10 - 17)  $\mu\text{mol/l}$  higher, UIBC was 23 (18 - 27)  $\mu\text{mol/l}$  lower, TIBC was 9 (5 - 13)  $\mu\text{mol/l}$  lower, transferrin saturation was 36 (31 - 41) percentage points higher, and hemoglobin was 0.9 (0.4 - 1.4) g/dl higher. There were no significant differences between H63D homozygotes and wild types. Serum ferritin concentrations were similar (ratio=0.9; 0.7 - 1.3). Serum iron was 2 (-1 - 5)  $\mu\text{mol/l}$  higher, UIBC was 2 (-2 - 6)  $\mu\text{mol/l}$  lower, TIBC was 0 (-4 - 3)  $\mu\text{mol/l}$  lower, transferrin saturation was 4 (-1 - 9) percentage points higher and hemoglobin was 0.2 (-0.2 - 0.7) g/dl higher.

### **Further discussion**

Total iron intake is somewhat higher than in the Dietary and Nutritional Survey of British Adults for women of similar age (12.9 mg/day).<sup>12</sup> The mean daily intake of heme iron is also considerably lower (1.0 mg/day).<sup>12</sup> This may be due to the large proportion of vegetarians and low proportion of meat-eaters in the Cohort or because different dietary assessment tools were used. Heme iron intake in this study is also lower than in the Framingham study (0.9 mg/day), though one might expect the US population of both men and women to consume larger quantities of red meat.<sup>13</sup>

Heme and non-heme iron are absorbed by different pathways. Previous research has shown no detectable association between total iron intake (dominated by non-heme iron) and iron status in individuals,<sup>14</sup> but that there is an association with heme iron intake and with red meat.<sup>2,15</sup> Our findings confirm these conclusions. Heme iron is substantially more available and provides up to half of absorbed dietary iron in iron-replete individuals.<sup>16</sup> In addition, it is heme iron absorption that is most affected in people with hemochromatosis.<sup>16</sup> If dietary strategies to reduce iron accumulation are to be used in addition to standard treatment such as phlebotomy, it is therefore reasonable that these should focus on limiting heme iron intake. However, serum ferritin is also increased in the acute phase response and we cannot exclude this as a cause of an increased ferritin concentration in some patients.

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