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ORIGINAL CONTRIBUTION

Effect of hereditary haemochromatosis genotypes and iron overload on other trace elements

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Abstract
Purpose Hereditary haemochromatosis is a common genetic disorder involving dysregulation of iron absorption. There is some evidence to suggest that abnormal iron absorption and metabolism may influence the status of other important trace elements. In this study, the effect of abnormal *HFE* genotypes and associated iron overload on the status of other trace elements was examined.
Methods Dietary data and blood samples were collected from 199 subjects (mean age = 55.4 years; range = 21–81 years). Dietary intakes, serum selenium, copper and zinc concentrations and related antioxidant enzymes (glutathione peroxidase and superoxide dismutase) in subjects with normal *HFE* genotype ($n = 118$) were compared to those with abnormal *HFE* genotype, with both normal iron status ($n = 42$) and iron overload ($n = 39$).
Results For most dietary and biochemical variables measured, there were no significant differences between study groups. Red cell GPx was significantly higher in male subjects with normal genotypes and normal iron status compared to those with abnormal genotypes and normal iron status ($P = 0.03$) or iron overload ($P = 0.001$). Red cell GPx was also highest in normal women and significantly lower in the abnormal genotype and normal iron group ($P = 0.016$), but not in the iron overload group ($P = 0.078$).
Conclusion Although it may not be possible to exclude a small effect between the genotype groups on RBC GPx, overall, haemochromatosis genotypes or iron overload did not appear to have a significant effect on selenium, copper or zinc status.

Keywords Copper · Selenium · Zinc · Trace element · Haemochromatosis · Iron overload

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Introduction

Hereditary haemochromatosis

Hereditary haemochromatosis (HH) is a common autosomal recessive disease. In Caucasian populations of northern European origin, one in 200 is homozygous for the major mutation [1]. This most important mutation, C282Y, the result of a single base transition, prevents correct membrane localisation of the *HFE* protein and hence affects the regulation of gastrointestinal iron absorption. A similar substitution, H63D, is common but confers a milder phenotype [2]. Mutations in non-*HFE* genes affecting the proteins ferroportin [3], hemojuvelin [4] and transferrin receptor 2 [5] are also known to cause haemochromatosis.

The penetrance and progression rate of haemochromatosis due to *HFE* mutations varies [6–9], but in susceptible people, dysregulated iron absorption continues despite sufficient iron stores and leads to an excessive accumulation of iron, oxidative stress and eventually parenchymal cell damage in many organs, particularly the liver [10]. Typically, high requirements for growth in adolescence, or loss through menstruation in women, means that despite abnormal iron absorption, excess iron storage generally does not become clinically significant until middle age [11]. Dietary, genetic and environmental factors are likely to be significant modifiers of the HH phenotype [12]. Absorption of iron occurs primarily in the duodenum where the major dietary

form, non-haeme iron, is reduced prior to uptake into the enterocyte [13]. Uptake is via a divalent metal transporter that appears to have a broad specificity. The dysregulated iron absorption associated with haemochromatosis could potentially inhibit or enhance the uptake of other important trace metals via this transporter, a resultant altered status possibly influencing the progression of the disease given the involvement of trace metals in many areas of metabolism, and in particular antioxidant protection [14, 15].

Relationships between iron and other trace elements

The divalent metal transporter 1 (DMT1) is an important duodenal transporter of Fe^{2+} and appears to also transport Cu, Cd, Mn, Pb, Co, Ni and Zn [16]. Iron repletion has been shown to down-regulate the expression of DMT1 and hence limit the absorption of other cations, such as Ni, Pb and Cd [17–20]. This effect, and a greater affinity for ferrous iron compared to other metals, may explain altered copper and zinc absorption in periods of high iron intake [21, 22].

DMT1 expression and activity is higher in HH despite iron repletion [23, 24]; a resultant increase in absorption of other trace elements could explain the increased tissue levels of non-ferrous metals such as Zn, Mn and Pb that have been observed previously in haemochromatosis [25]. Studies utilising experimental iron overload (in the absence of molecular defects) have also shown altered distributions and increases in body stores of Zn, Cd and Mn [26, 27], suggesting that other regulatory processes for metal absorption may be affected in states of iron overload.

Despite lacking a common absorption mechanism, there are some reports of associations between iron and selenium status. In animals, both iron overload and iron deficiency have been associated with decreased selenium status [28, 29], while selenium deficiency is linked with changes in tissue distribution of iron and other minerals [30, 31]. In limited human studies, iron supplementation in young women and in pregnant women appeared to negatively influence selenium status [32, 33].

Abnormal trace element status in conjunction with iron overload could potentially exacerbate the haemochromatosis phenotype given the increased oxidative stress experienced in iron overload conditions, and the importance of trace element-dependent enzymes such as glutathione peroxidase and superoxide dismutase in antioxidant defence. Given associations between iron and other minerals observed by others previously, we hypothesise that haemochromatosis could influence the status of other important trace elements. Therefore, the aim of this study was to investigate whether an abnormal *HFE* genotype alone, or in conjunction with abnormally high iron status, might influence serum levels of other trace elements such as selenium, copper or zinc or related antioxidant enzymes.

Subjects and methods

Ethics approval was provided by the Human Research Ethics Committee (Tasmania) Network, and the study was conducted according to the ethical guidelines laid down in the Declaration of Helsinki. The subjects for the study were 199 non-institutionalised male and female adults residing in north and northwest Tasmania, Australia. The majority of subjects ($n = 117$) were members of a database from the study 'The Influence of Diet, Health and Lifestyle Factors on Clinical Disease Development in the Tasmanian Population with Haemochromatosis Genes'. Other subjects without iron overload ($n = 82$) were from a convenience sample from the northern Tasmanian population; these subjects (normal genotype, $n = 75$; abnormal genotype, $n = 7$) were recruited at baseline from volunteers in other concurrent, population-based University of Tasmania studies and had similar age and gender profiles to the cohort from the Haemochromatosis database. Subjects provided informed written consent and completed a semi-quantitative food frequency questionnaire (FFQ) [34]. Data from Australian food composition tables [35] and responses from the FFQ were used to estimate daily selenium, copper and zinc intakes. Venous blood samples were collected into trace element-free serum, lithium heparin and EDTA Vacutainer tubes (Becton–Dickinson, Rutherford, USA).

Blood samples were separated by refrigerated centrifugation at $1{,}335 \times g$ for 15 min. Lithium heparin whole blood was used to produce a red cell lysate using the method of Abiaka and co-workers [36]. Lysate and serum were stored at −80 °C until analysis. Laboratory analyses included measurements of serum selenium, copper and zinc, as well as the selenium-dependent glutathione peroxidase (GPx) and CuZn-superoxide dismutase (SOD).

Analysis for GPx in serum and red cells (Ransel; Randox Laboratories Ltd., Crumlin, UK) was performed at 37 °C using a Data Pro clinical analyser (Thermo-Electron Corporation, Melbourne, Australia). SOD was measured in red cells using the Ransod assay (Randox Laboratories Ltd., Crumlin, UK) at 37 °C using the Cobas Mira autoanalyser (Roche Diagnostics Ltd., Rotkreuz, Switzerland).

Trace element analysis was performed in the Special Chemistry Laboratory at the Royal Hobart Hospital, Hobart, Australia. Serum selenium was determined by Zeeman-corrected graphite furnace atomic absorption spectrometry (GFAAS) using a Spectra 640Z spectrophotometer (Varian Inc., Palo Alto, USA) and the method of Saeed and colleagues [37]. Analysis of Seronorm Trace Elements control serum (Sero, Billingstad, Norway) with a certified selenium concentration of 0.92 µmol/l gave a mean of 0.88 µmol/l (CV; 7.0%; $n = 19$).

Serum copper and zinc concentration was determined by flame atomic absorption spectrometry (FAAS) using a

Spectra 880 spectrophotometer (Varian Inc., Palo Alto, USA) and the method of Meret and Henkin [38]. Analysis of Seronorm controls (Sero, Billingstad, Norway) with certified copper concentrations of 13.2 and 27.4 μmol/l gave means of 11.4 (CV; 3.8%; $n = 38$) and 23.8 μmol/l (CV; 3.2%; $n = 38$), respectively. For zinc, Seronorm controls with certified zinc concentrations of 14.0 and 16.1 μmol/l gave means of 13.0 (CV; 4.8%; $n = 38$) and 17.8 μmol/l (CV; 4.0%; $n = 38$).

Serum ferritin was determined using a two-site chemiluminescent immunometric assay (LKFE1) on the Immulite analyser (Siemens Healthcare Diagnostics; Deerfield, USA).

Genomic DNA for *HFE* genotyping was extracted from EDTA whole blood using the Blood and Tissue Genomic DNA Extraction Miniprep method (Viogene; Sunnyvale, USA).

DNA extraction used two hundred microliters of EDTA whole blood; eluted DNA samples were stored at −20 °C. The protocol for genotyping for C282Y and H63D mutations in the *HFE* gene was a multiplex amplification refractory mutation system (ARMS) based on the method of Baty et al. [39].

Statistical analysis

General linear modelling (GLM) with robust standard error estimation was used to test for any differences in trace element or antioxidant status between different groups as defined by gender, genotype and iron status (STATA version 9.2, StataCorp LP, USA). Post-estimation Holm test analysis was then used to adjust *P* values for multiple comparisons [40]. The validity of regression assumptions was tested by post hoc analysis to exclude significant heteroscedasticity and missing variable effects.

Results

Subjects had a mean ± SD age of 55.4 ± 12.9 years. Mean ± SD body mass index (BMI) was 26.8 ± 4.5 kg/m^2; 87 subjects were overweight, and 41 were classed as obese. Genotyping resulted in detection of six genotypes (Table 1): wild-type *HFE* homozygote, H63D heterozygote, H63D homozygote, C282Y heterozygote, C282Y/H63D compound heterozygote and C282Y homozygote. Study subjects were classified by serum ferritin levels (normal ferritin concentrations ≤300 μg/l in men and ≤200 μg/l in women), gender and *HFE* genotype producing three gender subgroups: group 1 (normal *HFE* genotype + normal iron status), group 2 (abnormal *HFE* genotype + normal iron status) and group 3 (abnormal *HFE* genotype + abnormal iron status) (Table 2). In this classification, heterozygote H63D subjects were considered to have a normal *HFE* genotype.

Between genders, there were significant differences in BMI, dietary intakes and serum copper concentrations (Tables 3 and 4). Amongst the male subject subgroups, there were no significant differences in age, energy intake or trace element intake. BMI did vary significantly amongst the male subgroups; men in group 3 had higher BMI compared to both group 1 ($P = 0.004$) and group 2 men ($P = 0.009$). In women, BMI and dietary intakes were not significantly different between groups. However, group 3 subjects were significantly older compared to group 1 ($P < 0.001$) and group 2 women ($P = 0.007$) (Table 3).

Few significant differences between groups were observed in the trace element and the related antioxidant enzymes (Table 4). In both men and women, only RBC GPx activity varied significantly between groups; with group 1 subjects of both genders having the highest activity levels of this protective antioxidant enzyme.

Discussion

Hereditary haemochromatosis (HH) appears to have a varied penetrance and rate of progression. In this disease, alterations in trace elements other than iron, particularly those with antioxidant function, could affect progression; however, there are few reports on the influence of HH genotype and iron overload on other micronutrients. The aim of this study was to investigate the status of the trace elements selenium, copper and zinc in subjects with abnormal *HFE* genotypes, in the presence and absence of abnormally high iron status.

The main finding of this study was that an abnormal *HFE* genotype, or increased iron status associated with an

Table 1 Distribution of *HFE* genotypes amongst study subjects

	HFE genotype					
	Homozygous C282Y	Compound Heterozygous C282Y/H63D	Heterozygous C282Y	Homozygous H63D	Heterozygous H63D	Normal 'wild-type' genotype
Male, *n*	10	11	15	1	11	39
Female, *n*	14	13	14	0	23	48
All subjects, *n*	24	24	29	1	34	87

Table 2 Ferritin levels of study subgroups

	Serum ferritin (μg/l)		
	Group 1 ($n = 118$)	Group 2 ($n = 42$)	Group 3 ($n = 39$)
Male	139.3 ± 78.9 (13–280)	155.0 ± 83.7 (12–288)	820.7 ± 547.1 (313–2,430)
Female	71.1 ± 50.5 (7–195)	77.0 ± 54.1 (7–193)	656.9 ± 565.5 (246–2,120)

Normal ferritin: *men* 30–300 μg/l, *women* 15–200 μg/l

Values are mean ± SD (range)

Group 1 normal *HFE* genotype and normal Fe status, *Group 2* abnormal *HFE* genotype and normal Fe status, *Group 3* abnormal *HFE* genotype and abnormal Fe status

Table 3 Age, BMI and dietary intakes in male and female subgroup subjects

	N	Age (years)	BMI (kg/m^2)	Energy (mJ)	Selenium (μg/day)	Copper (mg/day)	Zinc (mg/day)
Male							
Group 1	48	54.7 ± 13.4	26.7 ± 3.6	9.43 ± 3.68	77.3 ± 40.1	1.53 ± 0.60	12.8 ± 5.8
Group 2	16	55.0 ± 12.7	26.5 ± 3.3	11.27 ± 3.55	85.0 ± 27.1	1.59 ± 0.54	14.7 ± 3.9
Group 3	23	58.3 ± 10.8	29.5 ± 3.7[c,d]	9.72 ± 3.81	71.6 ± 30.7	1.33 ± 0.41	11.9 ± 3.9
Female							
Group 1	70	53.7 ± 13.3	26.1 ± 4.2	6.73 ± 2.08	63.5 ± 19.7	1.27 ± 0.38	10.3 ± 2.8
Group 2	26	54.2 ± 13.9	27.7 ± 5.9	6.43 ± 1.89	63.3 ± 35.9	1.22 ± 0.59	10.1 ± 3.1
Group 3	16	63.4 ± 8.1[a,b]	24.8 ± 5.6	7.52 ± 2.32	66.5 ± 24.7	1.58 ± 0.64	11.5 ± 4.1

Male versus female	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)
Group 1	−1.0 (−5.9, 3.8)	−0.4 (−1.9, 0.9)	−2.76 (−3.93, −1.60)**	−16.8 (−32.1, −1.5)*	−0.26 (−0.46, −0.05)*	−2.5 (−4.4, −0.7)*
Group 2	−0.8 (−9.1, 7.6)	1.2 (−1.7, 4.1)	−5.05 (−7.01, −3.04)**	−31.0 (−52.4, −9.7)*	−0.39 (−0.76, −0.03)*	−4.8 (−7.2, −2.3)**
Group 3	5.2 (−0.6, 10.9)	−4.8 (−7.8, −1.7)*	−2.23 (−4.29, −0.18)*	−6.1 (−28.2, 15.9)	0.19 (−0.17, 0.56)	−0.4 (−2.9, 2.1)

For comparison of nutrient intake in groups within genders values are mean ± SD, estimated using GLM adjusted for age and BMI

Comparison of groups between genders also estimated using GLM adjusted for age and BMI

Group 1 normal *HFE* genotype and normal iron status, *Group 2* abnormal *HFE* genotype and normal iron status, *Group 3* abnormal *HFE* genotype and abnormal iron status

* $P < 0.05$

** $P < 0.001$

[a] Group 3 versus group 1 mean difference 9.8 (95% CI 4.8–14.7; $P < 0.001$)

[b] Group 3 versus group 2 mean difference 9.2 (95% CI 2.5–15.9; $P = 0.007$)

[c] Group 3 versus group 1 mean difference 2.8 (95% CI 1.0–4.6; $P = 0.004$)

[d] Group 3 versus group 2 mean difference 3.0 (95% CI 0.8–5.2; $P = 0.009$)

abnormal *HFE* genotype, did not have a significant effect on most variables measured. Serum levels of selenium, copper and zinc generally followed dietary intake trends in each group and were within normal ranges; in addition, RBC SOD activity did not appear to be associated with dietary intake or serum levels of copper or zinc, which are components of the SOD enzyme.

Although the study was perhaps limited by the relatively small sample sizes of the genotype groups, the only statistically significant difference observed in the biochemical variables measured was in RBC GPx activity, which was significantly lower in men with abnormal genotypes with and without iron overload, and in women with abnormal genotype but normal iron status. The GPxs are selenium-dependent antioxidant enzymes; their activity increases with increasing selenium status, up to a selenium concentration of approximately 1.14 μmol/l [41, 42], at which point enzyme activity is maximised. These enzymes are therefore useful functional indicators of selenium status in populations with moderately low selenium levels, such as in Tasmania. In the current study, as expected, the differences in GPx activities in gender sub-groups were observed

Table 4 Serum and red cell trace element and antioxidant enzyme indices for male and female subgroup subjects

	N	Serum Se (μmol/l)	Serum Cu (μmol/l)	Serum Zn (μmol/l)	Serum GPx (U/l)	Red cell GPx (U/g Hb)	Red cell SOD (U/g Hb)
Male							
Group 1	48	1.11 ± 0.18	15.3 ± 2.7	13.2 ± 2.4	878 ± 114	47.8 ± 17.7	1,251 ± 318
Group 2	16	1.09 ± 0.14	16.0 ± 2.1	13.8 ± 2.4	792 ± 177	38.2 ± 13.3[a]	1,224 ± 288
Group 3	23	1.06 ± 0.16	14.8 ± 2.9	13.5 ± 1.8	841 ± 162	35.7 ± 11.5[b]	1,140 ± 396
Female							
Group 1	70	1.08 ± 0.19	19.0 ± 3.9	13.6 ± 3.0	861 ± 157	50.8 ± 19.2	1,274 ± 305
Group 2	26	1.07 ± 0.16	19.1 ± 4.7	12.9 ± 2.4	802 ± 146	38.6 ± 17.5[c]	1,181 ± 424
Group 3	16	1.12 ± 0.19	20.7 ± 3.8	13.3 ± 2.5	963 ± 271	45.4 ± 18.9	1,230 ± 329

Male versus female	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)	Mean difference (95% CI)
Group 1	0.01 (−0.06 to 0.06)	3.8 (2.6–4.9)**	0.4 (−0.7 to 1.4)	−12 (−60 to 37)	3.7 (−3.3 to 10.8)	39 (−80 to 157)
Group 2	−0.02 (−0.10 to 0.07)	2.8 (0.8–4.8)*	−0.8 (−2.4 to 0.8)	2 (−101 to 105)	−1.0 (−10.5 to 8.5)	−53 (−274 to 167)
Group 3	0.01 (−0.11 to 0.12)	6.6 (4.3–8.9)**	−0.1 (−1.6 to 1.4)	84 (−72 to 240)	4.9 (−5.9 to 15.8)	−3 (−247 to 241)

For comparison of groups within genders values are mean ± SD, estimated using GLM adjusted for age and BMI

Comparison of groups between genders also estimated using GLM adjusted for age and BMI

Group 1 normal *HFE* genotype and normal iron status, *Group 2* abnormal *HFE* genotype and normal iron status, *Group 3* abnormal *HFE* genotype and abnormal iron status

* $P < 0.05$

** $P < 0.001$

[a] Group 1 versus group 2 mean difference −9.8 (95% CI −18.4 to −1.2; $P = 0.03$)

[b] Group 1 versus group 3 mean difference −12.6 (95% CI −19.4 to −5.9; $P = 0.001$)

[c] Group 1 versus group 2 mean difference −12.9 (95% CI −20.8 to −5.1; $P = 0.016$)

to roughly reflect the differences in serum selenium levels. Due to the life span of red blood cells, red cell GPx activity is thought to be more reflective of longer-term selenium status than serum selenium and serum GPx [42], which may vary more acutely. These results suggest that a small, long-term difference in selenium status between groups may occur in these subjects.

The lack of significant differences in dietary measures was perhaps surprising given that iron overload subjects may be expected to consume modified diets to help reduce iron intake and absorption, therefore affecting the intake of other nutrients. Amongst men, those with abnormal iron did consume the lowest mean trace element intakes; however, women with abnormally high iron status had the highest mean intakes. The reason for this difference between genders is not clear, but it is potentially influenced by the limitations inherent in the collection of dietary intake estimates; women have been reported to under-report food intakes previously [43], while the overall accuracy of FFQ's for dietary assessments has also been debated [44].

The greater mean age of the women with iron overload was not unexpected and can be explained by the fact that iron loss associated with menstruation generally protects women with dysregulated iron absorption from developing iron overload; however, iron overload may develop post-menopausally [45].

The pathophysiology of iron overload associated with HH is mediated by reactive oxygen species (ROS) [46]. In iron overload, the iron binding capacity of transferrin becomes saturated leaving iron in a non-transferrin bound form and hence redox active [47]. This redox active iron catalyses the production of ROS through Fenton reactions and can lead to oxidative stress and subsequent damage to lipids, proteins and nucleic acids. Such oxidative damage in tissues contributes to the clinical manifestations seen in HH such as fibrosis and later, cirrhosis [45]. Antioxidant enzymes such as GPx, SOD and catalase are a primary defence mechanism for preventing damage by ROS; in iron overload, if activity of RBC GPx (a reflection of longer-term activity in tissue) was reduced, it could result in an increase in oxidative stress and hence influence progression of the disease.

Increased H_2O_2 levels, resulting from reduced GPx activity, could also play a role in increasing cellular iron uptake via its activation of the iron-responsive protein, IRP1, which acts to enhance transferrin receptor expression and therefore cellular iron uptake and decreases ferritin synthesis

[48, 49]. The potential importance of antioxidant enzymes in haemochromatosis is highlighted by recent studies that indicate that polymorphisms conferring reduced activity in antioxidant enzymes such as SOD and glutathione S-transferase P1 are associated with increased rates of cirrhosis and hepatocellular carcinoma in HH [50, 51].

In summary, the main differences observed in the current study related to trace element status were due to gender and dietary differences, and while it appeared the effect of haemochromatosis was not significant, it was not possible to exclude a relevant effect on GPx activity as this would require a larger study.

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Conflict of interest The authors declare that they have no conflict of interest.

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