Disordered vascular compliance in haemochromatosis

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ORIGINAL ARTICLE

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Abstract
Background A relationship may exist between body iron stores, endothelial dysfunction and overall cardiovascular risk.
Aims To compare vascular compliance, biochemical endothelial function and antioxidant status between patients with homozygous hereditary haemochromatosis and healthy controls.
Methods Haemochromatosis patients and healthy controls were recruited. Measures of vascular compliance were assessed by applanation tonometry. Serological markers of endothelial function (plasma lipid hydroperoxides, cell adhesion molecules), antioxidant levels (ascorbate, lipid soluble antioxidants) and high-sensitivity C-reactive protein (CRP) were also measured.
Results Thirty-five hereditary haemochromatosis patients (ten females, mean age 54.6) and 36 controls (27 female, mean age 54.0) were recruited. Haemochromatosis patients had significantly higher systolic and diastolic blood pressures. Pulse wave velocity (PWV) was significantly higher in male haemochromatosis patients (9.90 vs. 8.65 m/s, $p = 0.048$). Following adjustment for age and blood pressure, male haemochromatosis patients continued to have a trend for higher PWVs (+1.37 m/s, $p = 0.058$). Haemochromatosis patients had significantly lower levels of ascorbate (46.11 vs. 72.68 μmol/L, $p = 0.011$), retinol (1.17 vs. 1.81 μmol/L, $p = 0.001$) and g-tocopherol (2.51 vs. 3.14 μmol/L, $p = 0.011$). However, there was no difference in lipid hydroperoxides (0.46 vs. 0.47 nmol/L, $p = 0.94$), cell adhesion molecule levels (ICAM: 348.12 vs. 308.03 ng/mL, $p = 0.32$ and VCAM: 472.78 vs. 461.31 ng/mL, $p = 0.79$) or high-sensitivity CRP (225.01 vs. 207.13 mg/L, $p = 0.32$).
Conclusions Haemochromatosis is associated with higher PWVs in males and diminished antioxidants across the sexes but no evidence of endothelial dysfunction or increased lipid peroxidation.

Keywords Antioxidant · Arterial · Compliance · Haemochromatosis · Iron · Vascular

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Introduction

The potential link between body iron stores and cardiovascular risk (iron-heart hypothesis) was first described by Sullivan [1] during the assessment of serum iron levels between genders and its corresponding effects on cardiovascular risk. Salonen et al. [2] reported that serum ferritin concentrations ≥200 μg/L were associated with a >2-fold increased risk of acute myocardial infarction in men. Subsequent studies by Salonen et al. [3, 4] demonstrated that serum iron was a significant risk predictor for early atherogenesis, while Kiechl et al. identified serum ferritin as one of the strongest long-term predictors of carotid atherosclerosis progression. Additional support for these atherosclerotic associations is derived from studies on the Finnish population, who are known to have a high incidence of haemochromatosis, where the common C282Y mutation of the HFE gene is linked to increased cardiovascular risk [2, 5, 6].

Brissot et al. [7] reported vitamin C (ascorbate) deficiency in untreated idiopathic haemochromatosis and suggested that iron overload was the causative factor. Combined with increasing evidence of lipid peroxidation in association with iron excess in haemochromatosis patients, it is thought that a reduction in ascorbate levels may reflect consumption rather than malabsorption. Indeed, in the presence of raised iron stores, ascorbate assumes a pro-oxidant status [8]. Iron overload and haemochromatosis have also been associated with low levels of other antioxidants including vitamin E (alpha-tocopherol) and vitamin A (retinol) [9, 10]. Our own group has previously demonstrated that haemochromatosis patients have increased markers of lipid peroxidation in association with diminished levels of ascorbate, retinol and alpha-tocopherol [11].

Atherosclerosis is now considered to be an inflammatory disease of the blood vessel wall, characterised in early stages by endothelial dysfunction, recruitment and activation of monocytes, macrophages and dedifferentiation plus migration of vascular smooth muscle cells to later form the bulk of the atherosclerotic plaque [12, 13]. Although inflammation may initiate the disease, it is likely that the resulting oxidative stress propagates it and worsens injury [14].

Given the established association between atherosclerosis and vascular endothelial dysfunction, it seems possible that individuals with significant iron overload due to hereditary haemochromatosis will have endothelial dysfunction and altered arterial compliance [15, 16]. Due to limited evidence demonstrating endothelial dysfunction in association with haemochromatosis, we hypothesised that the significant iron overload associated with haemochromatosis correlates with altered arterial compliance and endothelial dysfunction.

The objectives of this study were to compare vascular compliance, biochemical endothelial dysfunction and antioxidant status between patients with homozygous hereditary haemochromatosis and healthy controls.

Materials and methods

Patient recruitment

Haemochromatosis patients aged between 20 and 75 years were recruited from the Liver Clinic at the Royal Victoria Hospital, Belfast. Haemochromatosis was defined by the phenotypic pattern of raised ferritin (at time of recruitment or during a recent out-patient department attendance) in combination with homozygosity for the C282Y HFE gene. Healthy control subjects were recruited from hospital staff and members of the public. Subjects with known hypertension (blood pressure >160/90 mmHg), diabetes mellitus, a history of cardiovascular disease and those taking lipid lowering agents or hormonal preparations were excluded from the study. Written informed consent was obtained from all subjects. Ethical approval was obtained from the regional ethics committee for Northern Ireland at Queens University, Belfast.

Patient assessment

Following an overnight fast, each subject's height and weight was recorded to calculate their body mass index (weight/height^2) followed by calculation of blood pressure in duplicate after a 15 min rest in the supine position.

Estimation of vascular compliance

The methodologies of pulse wave analysis (PWA) and pulse wave velocity (PWV) gated to the cardiac cycle have previously been described [17–19]. After the patient had rested in the supine position in a temperature controlled room for a minimum of 15 min, radial pulse wave analysis was recorded with a Millar tonometer and the Sphygmocor system model SCORPx, incorporating the pulse wave velocity system Model SCOR-Vx (SPC-301; Millar instruments and Atcor medical, Sydney, Australia). For pulse wave analysis, triplicate measurements were made with the patient in the supine position from the radial artery of the dominant arm and the average reading was calculated. The Sphygmocor analysis software automatically processed the radial artery waveform data and using a generalised transfer function generated measures of vascular compliance including augmentation index calibrated to 75 beats per minute (AgIx75), time to reflectance (TR), Buckberg's subendocardial viability ratio (SEVR) and ejection duration percentage (ED %). Calculation of the PWV was similar to PWA with the analysis gated to the

cardiac cycle with separate readings from the dominant radial artery (distal site) and ipsilateral carotid artery (proximal site). Carotid-radial PWV was measured rather than carotid-femoral due to ease of reproducibility and acceptability to patients.

Biochemical assessment

Blood Sampling

Peripheral venous blood samples, using a tourniquet for <2 min, were collected after subjects were sitting quietly for 5 min following the arterial compliance assessment. Baseline liver function tests, glucose, lipid profiles and iron studies were tested in haemochromatosis patients. Liver function tests, glucose, lipid profiles and iron studies were not tested in control subjects who were assumed to be healthy. Plasma samples for ascorbate were centrifuged immediately. Serum collected for all other assays was clotted for 15-min and then centrifuged. All samples were transferred to 2 mL tubes (Sarstedt, Ireland) and stored at −80 °C. All commercial assay analyses were performed according to the manufacturers' guidelines. Intra- and inter-assay coefficients of variation were within satisfactory limits according to the manufacturers' guidelines.

Plasma lipid hydroperoxides

These were measured spectrophotometrically using the Ferrous Oxidation-Xylenol Orange-version 1-assay (FOX 1) which was used to determine hydroperoxides (HPO) in the aqueous phase of serum. Hydroperoxides oxidise ferrous ions to ferric ions in dilute acids and the resultant ferric ions were then determined using ferric sensitive dyes as an indirect measure of hydroperoxide concentration.

Intracellular and vascular cell adhesion molecules

These were measured using commercially available ELISA kits from Eli-pair (Diaclone, Besançon, France).

Ascorbate

Concentrations were determined by the enzymatic oxidation of ascorbic acid and subsequent quinoxaline formation to generate a fluorescent derivative measured on the Cobas Fara centrifugal analyser as described by Vuillemier and Keck [20].

Lipid soluble antioxidants

These levels were measured using a high-performance liquid chromatography technique (HPLC) using diode array detection to assess retinol, γ-tocopherol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene according to the method of Craft et al. [21]. The detection limits for retinol and the tocopherols were 0.05 nmol/l, while 0.005 nmol/l was used for carotenoids.

High-sensitive C-reactive protein

This was measured with a latex-enhanced immunoturbidimetric assay (Randox Pharmaceuticals, Curmlin) using an ILab 600 biochemical analyzer and ILab 600 computer software (Instrumentation Laboratories, Warrington).

Statistical analysis

All data were recorded in Microsoft Excel (Redmond, WA) with final statistical analyses performed using the Statistical Program for Social Sciences (SPSS 15.0 for windows; SPSS Inc., Chicago, IL, USA). Normal distribution was assessed using both q–q plots and histograms. Differences between groups for normally distributed data were assessed using student's *t* test, whilst log transformations of non-normal data were expressed as a ratio of geometric means with interquartile range, *p* values and 95 % confidence intervals. Geometric means reported the central tendency of log transformed data and were selected to minimise the influence of extreme data points. In addition, results were adjusted for both age and sex using univariate analysis.

Results

Patient recruitment

Thirty-five hereditary haemochromatosis patients (10 females, mean age 54.6 ± 11.0 years) and 36 control subjects (27 female, mean age 54.0 ± 12.2 years) were

Table 1 Comparison of demographics by patient group expressed as mean ± standard deviation (SD) except for Body Mass Index (BMI) which is expressed as geometric mean with interquartile range (IQR)

	HH	Controls	*p*
Number	35	36	–
Sex (m/f)	25/10	9/27	–
Age	54.6 ± 11.0	54.0 ± 12.2	0.82
SBP (mmHg)	146.9 ± 23.4	135.7 ± 16.1	0.022
DBP (mmHg)	85.3 + 14.6	77.4 ± 9.8	0.002
BMI	28.3 (25.6–31.7)	28.3 (23.6–33.6)	0.97
Smokers	4	4	0.97

DBP diastolic blood pressure, *HH* hereditary haemochromatosis, *SDP* systolic blood pressure

Table 2 Comparison of haemodynamic and vascular compliance variables between HH patients and control subjects using the student's *t* test combined with age and gender adjusted results

	HH ($N = 35$) mean (SD)	Control ($N = 36$) mean (SD)	Mean difference (95 % CI)	*p*	Mean difference adjusted for age and gender (95 % CI)	*p*
HR (bpm)	68.79 (9.85)	65.72 (8.68)	3.07 (−1.32–7.46)	0.168	4.40 (−0.42–9.22)	0.073
SBP (mmHg)	146.89 (23.40)	135.72 (16.06)	11.16 (1.68–20.64)	0.022	10.08 (0.16–20.01)	0.047
DBP (mmHg)	85.26 (11.13)	77.36 (9.77)	7.90 (2.94–12.85)	0.002	7.90 (2.21–13.59)	0.007
PWV (m/s)	9.57 (1.56)	8.78 (1.08)	0.80 (0.16–1.43)	0.015	0.67 (−0.06–1.39)	0.070
AgIx75 (%)	22.99 (11.82)	25.71 (11.11)	−2.72 (−8.15–2.70)	0.321	2.49 (−2.49–7.46)	0.322
TR (ms)	141.79 (12.09)	141.27 (15.63)	0.52 (−6.04–7.08)	0.875	−5.17 (−11.06–0.71)	0.084
ED (%)	35.83 (3.85)	36.10 (3.77)	−0.27 (−2.07–1.54)	0.767	1.31 (−0.57–3.19)	0.168
SEVR (%)	149.58 (23.74)	146.50 (26.15)	3.08 (−8.75–14.92)	0.605	−7.48 (−19.92–4.96)	0.234

AgIx augmentation index, *CI* confidence interval, *DBP* diastolic blood pressure, *ED* ejection duration, *HR* heart rate, *IQR* interquartile range, *PWV* pulse wave velocity, *SBP* systolic blood pressure, *SEVR* subendocardial viability ratio, *TR* time to reflectance

recruited. There was no significant difference in baseline characteristics between the groups apart from systolic and diastolic blood pressures which were found to be significantly higher in the haemochromatosis group (SBP: 146.9 vs. 135.7 mmHg, $p = 0.022$ and DBP: 85.3 vs. 77.4 mmHg, $p = 0.002$) (Table 1). Mean baseline liver function tests, glucose and lipid profiles were normal for the haemochromatosis patients, while mean serum iron studies were elevated as expected (serum iron; 31.09 ± 11.53 mmol/L, Ferritin; 789.62 ± 1,384.32 μg/L, Transferrin saturation; 72.72 ± 6.17 %).

Arterial compliance

There was no significant difference in pulse wave analysis between haemochromatosis patients and control subjects (AgIx75: 22.99 vs. 25.71 %, $p = 0.32$; TR: 141.79 vs. 141.27 m/s, $p = 0.88$; SEVR: 149.58 vs. 146.50 %, $p = 0.61$; ED %: 35.83 vs. 36.10, $p = 0.77$) (Table 2).

Pulse wave velocity was significantly higher in male haemochromatosis patients when compared to normal control patients (9.90 vs. 8.65 m/s, $p = 0.048$), while no difference was identified between the female groups (8.82 vs. 8.80 m/s, $p = 0.97$. Following adjustment for age, systolic and diastolic blood pressures, male haemochromatosis patients continued to have a trend for higher PWVs with a mean difference between patient groups of +1.37 m/s (95 %CI −0.52 to 2.80 m/s, $p = 0.058$), whilst no difference was again identified between the two female groups −0.13 m/s (95 % CI −0.98–0.73 m/s, $p = 0.767$).

Endothelial function

There was no difference in lipid hydroperoxides (0.46 vs. 0.47 nmol/L, $p = 0.94$) or cell adhesion molecule levels (ICAM: 348.12 vs. 308.03 ng/mL, $p = 0.32$ and VCAM: 472.78 vs. 461.31 ng/mL, $p = 0.79$) between the haemochromatosis patients and normal controls (Table 3).

Antioxidant levels

After adjustment for age and gender, Haemochromatosis patients had significantly lower levels of ascorbate (46.11 vs. 72.68 μmol/L, $p = 0.011$). Retinol (1.17 vs. 1.81 μmol/L, $p = 0.001$) and γ-tocopherol (2.51 vs. 3.14 μmol/L, $p = 0.011$) were also significantly lower but there was no significant difference between the two groups for the other lipid soluble antioxidants (Table 3).

High-sensitivity C-reactive protein

There was no difference in high-sensitive CRP levels between the two groups (225.01 vs. 207.13 mg/L, $p = 0.32$) (Table 3).

Discussion

Previous researchers have described a relationship between body iron stores, endothelial dysfunction and overall cardiovascular risk [2–6, 15, 16]. We, therefore, hypothesised that the significant iron overload associated with haemochromatosis would correlate with reduced arterial compliance and endothelial dysfunction. Although the results of our study suggest a reduction in vascular compliance amongst male haemochromatosis patients through significantly elevated brachial PWVs, no change was identified in the female group. As hypertension is known to influence PWV, we subsequently adjusted our results to account for both patient age and blood pressure since baseline blood pressures were elevated in the haemochromatosis patients [22]. Although we continued to identify a trend for raised

Table 3 Comparison of markers of endothelial function and antioxidant levels between HH patients and control subjects using the student's *t* test combined with age and gender adjusted results following log transformation on those parameters not normally

	HH mean (IQR)	Control mean (IQR)	Ratio of geometric mean (GM) (95 % CI)	*p*	Ratio of GM adjusted for age and gender (95 % CI)	*p*
HPO (μmol/L)	0.46 (0.38–0.49)	0.47 (0.39–0.54)	0.96 (0.81–1.14)	0.65	0.99 (0.82–1.21)	0.94
HSCRP (μg/dL)	225.01 (136.79–347.32)	207.13 (101.31–305.04)	1.09 (0.77–1.53)	0.63	1.20 (0.83–1.74)	0.32
sICAM1 (ng/mL)	348.12 (256–522)	308.03 (255.5–402.50)	1.13 (0.89–1.44)	0.31	1.24 (0.95–1.61)	0.11
sVCAM1 (ng/mL)	472.78 (347–585)	461.31 (385.25–600.50)	1.03 (0.85–1.24)	0.80	0.97 (0.79–1.20)	0.79
α-carotene (μmol/L)	0.05 (0.03–0.09)	0.04 (0.02–0.08)	1.02 (0.70–1.48)	0.91	1.05 (0.68–1.62)	0.81
α-tocopherol (μmol/L)	25.60 (22.82–29.93)	27.26 (22.79–32.94)	0.94 (0.84–1.05)	0.25	0.97 (0.86–1.10)	0.68
β-carotene (μmol/L)	0.21 (0.15–0.32)	0.16 (0.09–0.29)	1.31 (0.95–1.80)	0.10	1.38 (0.95–2.01)	0.09
β-cryptoxanthin (μmol/L)	0.04 (0.25–0.84)	0.06 (0.31–0.82)	0.73 (0.50–1.06)	0.10	0.76 (0.48–1.18)	0.21
γ-tocopherol (μmol/L)	2.51 (1.97–3.12)	3.14 (2.47–3.81)	0.80 (0.69–0.92)	0.003	0.99 (0.68–0.95)	0.011
Lutein (μmol/L)	0.47 (0.09-0.16)	0.11 (0.07-0.17)	1.10 (0.87–1.38)	0.43	1.20 (0.86–1.46)	0.39
Lycopene (μmol/L)	0.33 (0.21-0.52)	0.34 (0.22-0.53)	0.95 (0.70–1.29)	0.76	1.04 (0.73–1.48)	0.82
Retinol (μmol/L)	1.17 (1.02-1.41)	1.81 (1.39-2.35)	0.64 (0.53–0.78)	<0.001	0.67 (0.52–0.84)	0.001
Zeaxanthin (μmol/L)	0.02 (0.01-0.04)	0.03 (0.02-0.05)	0.83 (0.62–1.11)	0.20	0.90 (0.64–1.25)	0.52

	HH mean (SD)	Control mean (SD)	Mean difference (95 % CI)	*p*	Mean difference adjusted for age and gender (95 % CI)	*p*
Vitamin C (μmol/L)	46.11 (34.83)	72.68 (39.18)	26.57 (8.77–44.67)	0.004	27.19 (6.44–47.94)	0.011

Student's *t* test analysis of Vitamin C comparing HH subjects with controls is reported separately

HSCRP high-sensitivity C-reactive protein, *sICAM* serum intracellular adhesion molecule, *sVCAM* serum vascular cell adhesion molecule

PWV in the male haemochromatosis patients, statistical significance was lost suggesting that this effect may not be absolute. A larger sample size with matched controls may, therefore, be required to definitively confirm or exclude this relationship.

Gaenzer et al. [16] have previously identified similar endothelial dysfunction and increased cardiovascular risk in male haemochromatosis patients particularly those not receiving venesection therapy. However, similar to our study and irrespective of treatment status, they identified no difference in vascular parameters between female haemochromatosis patients and control subjects. It was noted that none of the female patients had severe iron overload. Although not formally assessed in our study it is postulated that menstruation may confer a protective effect in female patients through lower overall iron loads.

The other possibility is that a relationship between haemochromatosis and reduced arterial compliance may not exist. For instance, there is conflicting evidence regarding the link between iron stores and cardiovascular risk from various population-based studies as well as a large individual patient meta-analysis [23–26]. A further study has even suggested possible cardiovascular protection in those with hemochromatosis associated with HFE C282Y homozygosity [27]. This is endorsed by a recent study by Valenti et al. [28] who demonstrated significantly increased vascular damage of the carotid artery in patients with non-alcoholic fatty liver disease that did not carry the HFE mutation. These findings could be explained by hepcidin up-regulation in those not carrying the HFE mutation but displaying iron overload, which may lead to iron compartmentalization into macrophages. Indeed, macrophages are a critical cell type involved in atherosclerosis and cardio-protection in haemochromatosis may also be explained by a mutational effect of selective iron depletion in these cells [29, 30].

Elevations in pulse wave velocity are known to correlate with reduced vascular compliance combined with associated serological endothelial dysfunction suggesting an increase in overall cardiovascular risk for that individual [31]. In fact, endothelial dysfunction has long been recognised as an important early functional abnormality and accepted surrogate marker of atherosclerosis [32, 33]. In haemochromatosis patients, lipid peroxidation is suggested as the most likely mechanism of iron-mediated atherosclerosis where iron may catalyse the formation of reactive oxygen species via the Fenton reaction leading to oxygen radical formation and subsequent lipid peroxidation [34, 35]. In contrast, Bozzini et al. [36] did not support the role for biochemical or genetic markers of iron stores as predictors of the risk of coronary atherosclerotic disease or its thrombotic complications through assessment of the relationships between ferritin and serum markers of either inflammation (CRP) or lipid peroxidation (malondialdehyde). Recent evidence also

suggests that the formation of hydroxyl radicals in iron overload, and a role for these molecules in both fibrin deposition and clot formation, which could be an important contributor to cardiovascular risk [37–41].

Although our study did not demonstrate any associated evidence of endothelial dysfunction in haemochromatosis patients through analyses of lipid hydroperoxides and soluble adhesion molecules, previous work by our own research group has demonstrated evidence of lipid peroxidation in association with haemochromatosis [11]. However, we acknowledge that the previous work had smaller patient numbers ($n = 15$) combined with the measurement of thiobarbituric acid reactive substances (TBARS) rather than lipid hydroperoxides as a surrogate marker of endothelial dysfunction. Gutteridge et al. [42] has also previously reported evidence of lipid peroxidation in association with haemochromatosis using TBARS. Although TBARS is a commonly used modality for assessment of lipid peroxidation, our unit and others have found it nonspecific and, therefore, we have switched to the assessment of lipid hydroperoxides using the Ferrous Oxidation-Xylenol Orange-version 1-assay [43].

While assessment of these serum markers of endothelial dysfunction cannot totally exclude iron generated oxidative stress in the vessel wall or an increase in cardiovascular risk, it is unclear whether such a deleterious effect is related to this or a different biochemical pathway such as hydroxyl radical formation. It is also unclear whether assessment of alternative investigative markers of endothelial function is warranted or simply that we need to recruit larger patient numbers for future studies [36, 44].

This study has additionally identified lower baseline levels of antioxidants in the haemochromatosis group when compared to normal controls. These results remain consistent with earlier findings where relative Vitamin C deficiency has been observed in association with haemochromatosis [8, 11]. Such a reduction in Vitamin C levels is thought to reflect consumption due to increased free radical formation in association with elevated free iron rather than malabsorption. Furthermore, this study also concurred with the findings of previous studies where reductions in other antioxidants (retinol and tocopherol) were identified as well [9–11]. This may reflect ongoing oxidative stress [10]. However, we would expect a subsequent increase in lipid peroxidation, which we did not identify [8].

Finally, high-sensitivity CRP is regarded as a predictor of future cardiovascular events and has previously been shown to correlate with raised serum ferritin concentration in patients with coronary atherosclerosis [36, 45, 46]. In this study, whilst an elevated high-sensitivity CRP was identified in the haemochromatosis patients, this difference was lost following correction for age and gender differences between groups.

The limitations of this study include the small number of participants and predominance of females in the control group, which could lead to an overestimation of vascular involvement in males with hereditary haemochromatosis. Patients from the haemochromatosis group were also not retested following iron depletion. Furthermore, a lack of baseline blood investigations in healthy controls is another major limitation of this study. Mean baseline liver function tests, glucose and lipid profiles were normal for the haemochromatosis patients, while serum iron studies were elevated as expected. These parameters were not measured in control subjects who were presumed healthy recruits. In addition, control subjects were not tested for HFE gene mutations. As previously discussed, future research strategies could target larger patient numbers, the impact of venesection and the use of alternative or combination modalities to assess endothelial function including lipid hydroperoxides, TBARS as well as urinary isoprostanes.

Conclusion

In haemochromatosis, male patients have reduced arterial compliance combined with diminished antioxidant levels across the sexes. However, there is no associated serological evidence of endothelial dysfunction, increased lipid peroxidation or alteration in hsCRP. Further, larger studies are required to clarify these relationships and their correlation with future cardiovascular risk.

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