Alterations in sympathetic nerve traffic in genetic haemochromatosis before and after iron depletion therapy: a microneurographic study

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Aims

Haemochromatosis (HH) displays a number of circulatory alterations concurring at increase cardiovascular risk. Whether these include sympathetic abnormalities in unknown.

Methods and results

In 18 males with primary HH (age: 42.3 ± 10.4 years, mean ± SD), clinic and beat-to-beat blood pressure (BP, Finapres), heart rate (HR, EKG), and muscle sympathetic nerve activity (MSNA, microneurography) traffic were measured in the iron overload state and after iron depletion therapy. Haemochromatosis patients displayed elevated serum iron indices while other haemodynamic and metabolic variables were superimposable to ones seen in 12 healthy subjects (C). Muscle sympathetic nerve activity was significantly greater in HH than C (64.8 ± 13.3 vs. 37.8 ± 6.7 bs/100 hb, P < 0.01). Iron depletion caused a significant reduction in serum ferritin, transferrin saturation, and MSNA (from 64.8 ± 13.3 to 39.2 ± 9.2 bs/100 hb, P < 0.01) and a significant improvement in baroreflex-MSNA modulation. This was paralleled by a significant increase in the high-frequency HR variability and by a significant reduction in the low-frequency systolic BP variability components. Before after iron depletion therapy, MSNA was significantly and directly related to transferrin saturation, liver iron concentration, and iron removed, while the MSNA reductions observed after the procedure were significantly and inversely related to the baroreflex-MSNA increases detected after iron depletion. In C, all variables remained unchanged following 1 month observation.

Conclusion

These data provide the first evidence that in HH iron overload is associated with an hyperadrenergic state and a baroreflex alteration, which are reversed by iron depletion. These findings underline the importance of iron overload in modulating sympathetic activation, possibly participating at the elevated cardiovascular risk reported in HH.

Keywords

Haemochromatosis • Iron overload • Serum ferritin • Cardiovascular risk • Iron depletion • Sympathetic nervous system

Introduction

Iron overload carries an increase in cardiovascular risk¹–⁶ and its occurrence is associated with alterations in a spectrum of cardiovascular functions, such as an endothelial impairment, an alteration in oxidative stress and a proatherogenic and prothrombotic effect.⁷–¹¹ The alterations might also include an activation of the sympathetic cardiovascular drive, because in experimental animals iron (i) is involved in the modulation of central nervous system receptors for dopamine, i.e. a precursor of the adrenergic neurotransmitter norepinephrine¹²,¹³ and (ii) impairs the ability of reflexogenic areas anatomically located in the cardiac chambers as well as in the pulmonary circulation to inhibit central adrenergic outflow.¹³,¹⁴

No information is available as to whether and to what extent iron overload is associated with an increase in sympathetic cardiovascular drive in humans. The issue is not only of pathophysiological but also of clinical relevance, because in several cardiovascular and non-cardiovascular disease sympathetic neural outflow shows a direct independent relationship with cardiovascular and all-cause mortality,¹⁵–¹⁸ which implies that its activation might be involved

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in the increased cardiovascular risk reported in the iron overload state.

We set out to address this issue by directly quantifying effenter postganglionic muscle sympathetic nerve traffic via the micro-neurographic technique in patients affected by high iron Fe (HFE)-related haemochromatosis (HH), i.e. the most common human model of primary iron overload. The study was also designed to determine whether the hypothesized sympathetic overdrive characterizing the iron overload state could be favourably affected by iron depletion therapy and the mechanisms potentially responsible for this effect. The results of this second part of the protocol were thought to be crucial for strengthening the cause–effect nature of the relationship possibly found in the first part.

Methods

Population

Our study was carried out in 18 males aged between 26 and 58 years recruited from the patients referred to the centre for the diagnosis and treatment of HH of the San Gerardo Hospital in Monza, Italy. Patients were enrolled if characterized by (i) serum ferritin > 300 μg/L; (ii) the presence of hepatic iron overload with a liver iron concentration (LIC) by magnetic resonance imaging (MRI) > 100 μmol/g, and (iii) p.C282Y homozygous or mutations or p.C282Y/H63D compound heterozygous HFE genotypes. Patients with serum ferritin > 1000 μg/L and/or increased serum transaminases underwent liver biopsy for the assessment of liver fibrosis according to American Association for the Study of Liver Disease guidelines. Exclusion criteria were the presence of severe liver fibrosis or cirrhosis, all the secondary or acquired forms of iron overload (major thalassemia and congenital or acquired dyserythropoietic anaemia), previous treatment with i.v. iron supplements or repeated blood transfusions, viral or autoimmune chronic liver diseases, alcohol intake 30 g/day, and porphyria cutanea tarda. Very rare hereditary forms of iron overload like aceruloplasminemia and hereditary hypotransferrinaemia were also excluded. All patients were in sinus rhythm and none had a recent history of cigarette smoking. Coronary heart disease, congestive heart failure, cerebrovascular disease, renal insufficiency, obesity, hypertension, diabetes mellitus, liver cirrhosis, metabolic syndrome, obstructive sleep apnoea, respiratory diseases, polyneuropathy, or other conditions known to affect neuroadrenergic function were ruled out on the basis of anamnestic evidence, clinical evidence, or appropriate biochemical or instrumental work-up. Patients were under no drug treatment and all were studied on an outpatient basis. The protocol of the study was approved by the Ethics Committee of the Istituto Auxologico Italiano, Milan and of the IRCCS Multimedica, Sesto San Giovanni (Milan), Italy. All participants gave written consent to the study after being informed of its nature and purpose.

Measurements

Measurements include body mass index, waist circumference, waist-to-hip ratio, sphygmomanometric blood pressure (BP), beat-to-beat finger BP (Finapres, Ohmeda 2003, Englewood, FL, USA), heart rate (HR, EKG), and respiration rate (pneumotachograph). They also include (i) multunit recordings of MSNA via the microneurographic technique, as previously described; (ii) venous blood sample for laboratory tests which included lipid profile (total cholesterol), HDL-cholesterol or c-HDL, and triglycerides), plasma glucose, plasma insulin, homeostatic model assessment (HOMA) index [calculated via the formula plasma glucose (mmol/L) × serum insulin (μU/mL)/22.5], hepatic indices (aspartate aminotransferase-AST, alanine aminotransferase-ALT), complete blood count, and serum iron profile (serum iron, transferrin, ferritin; transferrin saturation calculated via the formule serum iron/serum transferrin/1.42 × 100); (iii) venous plasma norepinephrine measured by high-performance liquid chromatography; (iv) LIC evaluated through MRI (1.5 T) based on Gandon’s algorithm, with reference values < 36 μmol/g for physiologic condition, <100 μmol/g for mild iron overload, and >300 μmol/g for severe iron overload; (v) serum hepcidin-25 levels measured by ultra-high-pressure liquid chromatography-mass spectrometry; (vi) liver fibrosis assessed according to Ishak and coworkers; (vii) calculation of total iron removed by weekly or biweekly phlebotomies (400 mL) until iron depletion 81 mL of blood corresponding to 0.5 mg of iron; (viii) molecular analysis performed on genomic DNA extracted from peripheral leucocytes using polymerase chain reaction to identify p.C282Y and p.H63D mutations; and (ix) an echocardiographic evaluation of left ventricular ejection fraction, measured from the four-chamber apical projection by using the product area times length and left ventricular mass index, and calculated by the Devereux’s formulae normalized to body surface area.

Blood pressure, EKG, and MSNA were digitized with a sampling frequency of 1000 Hz (PowerLab recording system model ML870 8/30; AD Instruments, NSW2153, Australia). Muscle sympathetic nerve activity was quantified over a 20 min period as bursts incidence over time (bursts per minute), as bursts incidence corrected HR values (bursts per 100 heart beats) and as total activity (number of bursts per minute times mean burst amplitude, expressed in arbitrary units). The muscle nature of MSNA was assessed according to the criteria outlined in previous studies and the recordings were considered acceptable if the signal-to-noise ratio exceed the value of 3. Respiration rate was monitored by a strain gauge pneumograph positioned at midchest level. Heart rate and systolic BP variability were measured via the spectral analysis technique. Briefly, time series of BP and pulse pressure interval values were analysed by a parametric spectral estimation method based on an autoregressive model. Baroreflex control of MSNA was determined by a method similar to that described by Kienbaum and coworkers, as reported in a recent paper by our group. Briefly, the method allows to relate each spontaneous sympathetic burst to the diastolic BP and the cardiac interval during which the burst was generated.

A group of 12 healthy subjects performed two sessions spaced each other by 1 month interval without any therapeutic intervention and thus served as control.

Protocol and data analysis

Following iron status assessment, abdominal MRI and molecular analysis, candidates underwent an echocardiographic evaluation. The day following the echocardiographic examination, all patients came to the laboratory in the morning, after a light breakfast and an overnight abstinence from alcohol and coffee consumption. They were placed supine and fitted with the intravenous cannula and the devices to measure finger BP, HR, and respiration rate. After a 30-min interval, a blood sample for the assay of plasma norepinephrine was drawn from the cannula. After a 45-min interval, BP, HR, respiration rate, and MSNA were continuously measured. Data were collected in a semidark and quiet room at a constant temperature of 20–22 °C. The same protocol was performed at least 30 days after complete iron depletion treatment obtained through custom bloodletting sessions aimed to reduce serum ferritin between 50 and 100 μg/L. This temporal window was selected to avoid interference related to the cardiovascular and haematological consequences linked to iron depletion procedure. The control group performed its
two study sessions according to the same procedure. Data were ana-
lysed by a single investigator (R.D.) not involved in data collection
and unaware of patient status and study design. Values from individual sub-
jects were averaged for each group and expressed as means ± SD.
Comparisons between groups were made by t-test for unpaired obser-
vations while comparisons between sessions were made by t-test for
paired observations. The relationships between resting MSNA and
other parameters were investigated by linear regression analysis. Pear-
son product moment correlation coefficients (r) were calculated. A
two-sided \( P < 0.05 \) was taken as the level of statistical significance. All
analyses were performed with SAS software version 9.4 (SAS Institute
Inc., Cary, NC, USA).

Results

As shown in Table 1, HH patients and controls had a similar age, body
weight, body mass index, and waist-to-hip ratio. This was the case also
for BP and HR values. Haemochromatosis patients displayed a normal
lipid profile and showed plasma glucose, insulin, and HOMA index le-
vels within the normal range. Three patients showed an increase in
serum ALT, displaying, however, only a mild portal fibrosis at the bi-
opsy performed before the study. Fifteen patients showed a moder-
ate iron overload while the remaining two showed an LIC ≥
300 μmol/g. Serum hepcidin values were within the normal age-range
reported in the literature. Table 1 also shows that the majority (n =
15, 83%) of HH patients were free from metabolic alterations, such as
diabetes mellitus or glucose intolerance, hypercholesterolaemia or
hypertriglyceridaemia, overweight or obesity, and hepatic steatosis,
thus ruling out the presence of the metabolic syndrome. As shown
in Figure 1, MSNA values, expressed as bursts incidence over time,
bursts incidence corrected for HR, and total bursts amplitude,
were significantly greater in HH patients than in healthy controls.
This was accompanied by the finding that high-frequency HR variabil-
ity was significantly reduced in HH patients while low-frequency sys-
tolic BP variability significantly increased when compared with
controls (Table 2). This was not the case for venous plasma norepin-
ephrine, whose values were similar in the two groups (Table 2). Bar-
oreflex modulation of MSNA was significantly reduced in HH patients
when compared with controls (Table 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control subjects Before</th>
<th>After 30 days</th>
<th>HH patients Before</th>
<th>After iron depletion</th>
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<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>12</td>
<td>18</td>
<td>18</td>
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<tr>
<td>Age (years)</td>
<td>43.1 ± 6.6</td>
<td>42.3 ± 10.4</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.9 ± 1.6</td>
<td>24.6 ± 2.0</td>
<td>24.6 ± 2.0</td>
<td>25.1 ± 2.17</td>
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<td>WC (cm)</td>
<td>90.0 ± 10.3</td>
<td>90.0 ± 9.7</td>
<td>90.0 ± 9.7</td>
<td>90.9 ± 9.2</td>
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<tr>
<td>Clinic SBP (mmHg)</td>
<td>131.8 ± 7.8</td>
<td>131.9 ± 11.7</td>
<td>131.9 ± 11.7</td>
<td>128.1 ± 8.8</td>
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<tr>
<td>Clinic DBP (mmHg)</td>
<td>73.9 ± 8.9</td>
<td>72.0 ± 6.8</td>
<td>72.0 ± 6.8</td>
<td>70.6 ± 8.6</td>
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<td>Finger SBP (mmHg)</td>
<td>129.7 ± 8.0</td>
<td>129.1 ± 7.7</td>
<td>129.1 ± 7.7</td>
<td>130.1 ± 9.6</td>
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<tr>
<td>Finger DBP (mmHg)</td>
<td>70.5 ± 7.9</td>
<td>70.0 ± 7.8</td>
<td>70.1 ± 7.8</td>
<td>68.8 ± 7.9</td>
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<td>Heart rate (bpm)</td>
<td>70.0 ± 6.5</td>
<td>71.4 ± 6.9</td>
<td>69.6 ± 11.2</td>
<td>69.8 ± 10.7</td>
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<td>LVEF (%)</td>
<td>64.0 ± 2.2</td>
<td>62.8 ± 2.3</td>
<td>62.8 ± 2.3</td>
<td>62.7 ± 2.5</td>
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<td>E/A (a.u.)</td>
<td>1.27 ± 0.4</td>
<td>1.31 ± 0.5</td>
<td>1.31 ± 0.5</td>
<td>1.30 ± 0.4</td>
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<td>LVM (g/m²)</td>
<td>91.2 ± 4.4</td>
<td>89.9 ± 14.5</td>
<td>90.9 ± 15.0</td>
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<tr>
<td>Total cholesterol (mg/dL)</td>
<td>198.6 ± 36.2</td>
<td>181.3 ± 27.4</td>
<td>195.2 ± 24.1</td>
<td></td>
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<tr>
<td>HDL-cholesterol (mg/dL)</td>
<td>49.4 ± 18.2</td>
<td>55.0 ± 15.9</td>
<td>58.9 ± 18.5</td>
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<tr>
<td>Triglycerides (mg/dL)</td>
<td>107.0 ± 52.1</td>
<td>100.7 ± 46.4</td>
<td>94.8 ± 37.3</td>
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<tr>
<td>Plasma glucose (mg/dL)</td>
<td>88.5 ± 9.02</td>
<td>95.5 ± 8.0</td>
<td>96.3 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>Plasma insulin (μU/mL)</td>
<td>7.4 ± 1.4</td>
<td>7.8 ± 1.3</td>
<td>7.9 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>HOMA index (a.u.)</td>
<td>1.3 ± 0.41</td>
<td>1.6 ± 0.32</td>
<td>1.9 ± 0.39</td>
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<tr>
<td>ALT (U/L)</td>
<td>24.6 ± 3.3</td>
<td>31.9 ± 17.8</td>
<td>30.7 ± 14.2</td>
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</tr>
<tr>
<td>γGT (U/L)</td>
<td>23.5 ± 2.6</td>
<td>31.4 ± 29.0</td>
<td>28.3 ± 16.6</td>
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<tr>
<td>Haemoglobin (g/dL)</td>
<td>14.8 ± 0.2</td>
<td>15.0 ± 0.9</td>
<td>14.9 ± 0.7</td>
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</tr>
<tr>
<td>Haematocrit (%)</td>
<td>43.8 ± 1.8</td>
<td>43.4 ± 2.7</td>
<td>43.4 ± 2.2</td>
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</tr>
<tr>
<td>Serum ferritin (μg/L)</td>
<td>62.7 ± 24.2</td>
<td>730.8 ± 255.0</td>
<td>579.0 ± 213.21</td>
<td></td>
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<tr>
<td>Transferrin saturation (%)</td>
<td>n.a.</td>
<td>51.8 ± 17.1</td>
<td>37.4 ± 15.21</td>
<td></td>
</tr>
<tr>
<td>Hepcidin (ng/mL)</td>
<td>n.a.</td>
<td>11.56 ± 6.6</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>LIC (μmol/g)</td>
<td>n.a.</td>
<td>201.2 ± 57.2</td>
<td>n.a.</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as means ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; ALT, alanine aminotransferasi; γGT, γ-glutamil-transferasi; LVEF, left ventricular ejection fraction; LVM, left ventricular mass index; LIC, liver iron concentration; a.u., arbitrary units; n.a., not assessed. Asterisks (⁎⁎ P < 0.01) refer to the statistical significance vs. controls; (†† P < 0.01) vs. HH before iron depletion.
The effects of iron depletion on the variables included in the study are shown in Tables 1 and 2 as well as in Figures 2 and 3. Iron depletion did not significantly affect body weight, BP, HR, blood cells count, plasma lipid, and glucose profile (Table 1), but it caused, as expected, a marked and significant reduction in serum ferritin and transferrin saturation (Figure 2), the amount of the total iron depleted during phlebotomy being quantified as 3.0 ± 1.8 g during an average time period of 10.0 ± 4.3 months. As shown in the average data of Figure 2, in the original recordings of Figure 3 as well as in the individual data of the supplemental table, iron depletion was accompanied by a marked and significant reduction in MSNA, whose values after phlebotomy became almost superimposable to the ones seen in healthy control subjects. This was paralleled by a significant increase in high-frequency HR variability and a significant reduction in low-frequency systolic BP variability, with no significant changes in plasma norepinephrine values (Table 2). Iron depletion caused a significant improvement in baroreflex-MSNA sensitivity, whose values however remained lower than the ones characterizing controls (Table 2). Transferrin saturation values were significantly and directly related to MSNA when expressed as number of bursts corrected for HR both in the iron overload state and after iron depletion therapy (Figure 4, upper panels). Also LIC and iron removed significantly correlated with MSNA values corrected for HR (Figure 4, lower panels). MSNA reductions observed after the procedure were significantly and inversely related to the baroreflex-MSNA increases detected after iron depletion (Figure 5). In contrast, no correlation was detectable between serum ferritin and MSNA (Figure 4, upper panels), serum hepcidin, HOMA index, and MSNA values (data not shown). No significant change in all the measured variables was found in the control group after the 1 month observational period (Tables 1 and 2). In controls, no relationship was detected between MSNA values and the various variables of iron metabolism.

**Discussion**

Our study provides information on the relationships between iron overload and sympathetic cardiovascular drive in human beings that...
was not available before. First, patients affected by HH-related iron overload are characterized by a marked sympathetic overdrive whose magnitude appears to be directly related to LIC, iron removed, and serum transferrin saturation, i.e. a marker of the severity of the iron overload state. Second, the increase in adrenergic cardiovascular drive detectable in the iron overload state can be almost completely reversed by an iron depletion procedure capable to bring serum transferrin and serum iron content back to normal values. Third, the reduction of the adrenergic overdrive achievable with iron depletion procedure is directly related to the effect of the procedure on transferrin saturation. Taken together, these findings support the conclusion that (i) an activation of sympathetic circulatory drive should be recognized as an additional abnormality of HH and (ii) this abnormality closely reflects the iron load status and its reversibility with treatment.

Several other results of our study deserve to be discussed. First, in our HH patients the marked elevation in MSNA was accompanied by an increase in the low-frequency oscillations of systolic BP variability, which reflects the adrenergic tone in the whole cardiovascular system. This suggests that the augmented sympathetic vascular drive detected in HH does not only selectively affect the muscle circulation but it extends to other cardiovascular districts as well. Second, in our patients with HH-related iron overload, we found that the sympathetic activation was coupled with a reduced high-frequency component of HR variability, indicating an impaired cardiac parasympathetic modulation. This means that both the
components of cardiovascular autonomic function appear to be affected by the iron overload state. Third, both the sympathetic and the parasympathetic abnormalities do not appear to be irreversible, since the iron depletion procedure was capable to almost normalize adrenergic and vagal modulation of the cardiovascular system. Finally, in HH patients of the present study HR values (which depend on both sympathetic and parasympathetic influences on sinus node) did not differ from those detected in healthy controls, despite the marked alterations in autonomic balance observed in our patients with iron overload. This finding confirms that, as reported in other cardiovascular or cardiometabolic diseases, resting HR fails to provide any insight on the autonomic modulation of the cardiovascular system.

Our study was not designed to investigate the mechanisms responsible for the adrenergic overdrive seen in HH patients as well as for the sympathoinhibitory effects of iron depletion therapy. However, we can rule out that the increase in MSNA seen in these patients depends on the presence of a metabolic syndrome or an insulin resistance state, because metabolic syndrome was an exclusion criterion for the study population, which also displayed normal HOMA index values within the normal range. We can also rule out a causative role of hypertension or diabetes mellitus, i.e. clinical conditions characterized by a marked sympathetic overdrive, since our patients exhibited normal BP and plasma glucose values. We can finally exclude that the neuroadrenergic alteration was due to the presence of structural or functional alterations of the}

**Figure 4** Upper and middle panels: relationships between serum ferritin, transferrin saturation, and muscle sympathetic nerve activity, expressed as number of bursts corrected for heart rate (bs/100 hb), before (baseline, left panels), and after (iron depletion, right panels) iron depletion therapy. Note that only transferrin saturation was significantly and directly related to muscle sympathetic nerve activity both before and after iron depletion. Lower panels: relationships between hepatic iron, iron removed by phlebotomy, and muscle sympathetic nerve activity values. Pearson product moment correlation coefficients (r) and P-values are shown for each correlation.
heart known ‘per se’ to favour a hyperadrenergic state, since echocardiographic examinations excluded the presence of heart failure, diastolic dysfunction, and cardiac hypertrophy.\textsuperscript{13,21} We can, on the other hand, speculate that the sympathetic activation seen in our patients has a reflex origin, namely that it depends on a reduction of the restraint physiologically exerted by arterial baroreceptors on the sympathetic nervous system.\textsuperscript{11} This possibility is supported by the close significant correlation between the reduction in MSNA observed after iron depletion and the concomitant improvement in baroreflex-MSNA control. A further mechanism is that iron may play a role on the sympathetic neural function and we suggest that this effect might be mediated by an iron-induced oxidative stress. This is supported by the modulation of sympathetic activity induced by iron depletion and the significant correlations between MSNA and transferrin saturation, LIC and iron removed we observed in the present study. Indeed, it is well known that iron overload leads to formation of free iron (non-transferrin bound iron [NTBI] or labile iron pool [LIP]) in blood and cells and that NTBI and LIP directly correlated with transferrin saturation (essentially when it exceeds 75%) and amount of iron overload.\textsuperscript{27,34} Free iron catalyses redox reactions leading to oxidative stress reaction formation, lipoperoxidation, and cellular damage. Previous studies have shown that oxidative stress reaction can directly and functionally interact with sympathetic neurons through alterations in ion channels function and nitric oxide crowding.\textsuperscript{8,35} Furthermore, nitric oxide has been shown to affect central sympathetic transmission both directly and indirectly, i.e. via a vasodilatory-induced reflex activation.\textsuperscript{35}

Our study has some limitations and a clinical implication. One limitation is that our findings refer to the iron overload of genetic HH and thus they cannot safely extrapolated to other conditions characterized by an iron accumulation in blood and tissues, such as secondary HH. A second limitation is that we could not assess blood NTBI and indices of oxidative stress reaction activity to confirm a pathogenetic hypothesis. The clinical implication is that the activation of the sympathetic cardiovascular influences that occurs in HH may represent one of the pathophysiological mechanisms responsible for the increased cardiovascular risk reported in this disease,\textsuperscript{1,2,5,9,20} as it has been shown in other diseases characterized by an adrenergic overdose.\textsuperscript{15–18}

### Conflict of interest
none declared.

### References


