

Original article

Increase in type II collagen turnover after iron depletion in patients with hereditary haemochromatosis

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Abstract

Objective. To determine the effects of iron depletion on serum levels of joint biomarkers and on joint symptoms in patients with hereditary haemochromatosis (HH).

Methods. Levels of biomarkers were measured in 18 patients with HH at the time of diagnosis and after iron depletion. The markers were type II collagen degradation (Coll2-1) and its nitrated form (Coll2-1NO₂), type II procollagen synthesis (CPII), MPO, COMP and HA. For each patient, demographic data were collected and the global joint pain (visual analogue scale) was assessed before and after iron depletion by phlebotomy.

Results. A total of 18 patients [10 males; mean (s.d.) age 48 (11) years] were homozygous for the C282Y mutation. No patient had liver dysfunction. Ferritin level before iron removal was 627.5 (range 133–3276) µg/l, and duration of the iron depletion phase was 295 (70–670) days. Serum levels of both Coll2-1 and CPII were significantly increased from diagnosis after iron depletion: 80.1 (55.6–113.5) vs 96.0 (48.8–136.3) nM ($P=0.004$) and 731.4 (374.2–1012.3) vs 812.8 (535.8–1165.6) ng/ml ($P=0.03$), respectively. Levels of other biomarkers were not modified by iron depletion. Ferritin level, which at baseline was correlated with body iron store ($r=0.63$; $P=0.008$), was significantly correlated with HA level measured before iron depletion ($r=0.60$; $P=0.01$). Global joint pain was not correlated with ferritin concentration and did not significantly decrease after iron depletion: 43 (19–73) vs 36 (16–67) mm ($P=0.07$).

Conclusions. In patients with HH, cartilage homeostasis is modified by iron excess and an increase in type II collagen turnover occurs after excess iron removal.

Key words: Hereditary haemochromatosis, Cartilage, Biomarkers, Phlebotomy, Iron.

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Introduction

The most common form of hereditary haemochromatosis (HH) is an autosomal recessive iron-overload disorder associated with a mutation in the haemochromatosis (HFE) gene that results in the substitution of a tyrosine for a cysteine at amino acid 282 (C282Y). Increased iron absorption and iron overload in HH is due to deficiency of hepcidin, which plays a central role in the regulation of iron homeostasis [1].

Although homozygosity for the C282Y mutation is found in approximately 1 in 200 people of northern European

descent, the phenotypic expression of this mutation is highly variable [2–4]. Most homozygote patients have only elevated levels of ferritin and transferrin saturation, whereas 30% have iron overload-related diseases, defined as liver fibrosis, cirrhosis, hepatocellular carcinoma and arthropathy that frequently involves the second and third MCP joints [5].

Patients with HH report a wide range of rheumatological complaints [6, 7]. Affected patients may present with arthralgia in hands and large joints or severe arthropathy with structural damage that might seriously affect their quality of life [6–8]. Most often, this arthropathy mimics OA but with a different joint distribution and radiographic appearance [9]. MCP joints, wrists and knees are preferentially affected, and chondrocalcinosis is present in 10–30% of patients [10, 11].

The mechanism whereby iron overload produces joint pain and structural damage is not well understood. Iron deposits have been observed in cartilage and synovia from patients with HH, and iron may decrease proteoglycan synthesis by chondrocytes and down-regulate prostaglandin E2 production by fibroblasts [6, 7, 12]. However, the effect of iron excess on type II collagen turnover has never been investigated.

Phlebotomy therapy to remove excess iron is the cornerstone of treatment in HH. Some clinical features such as fatigue, skin pigmentation and insulin requirements are improved with phlebotomy, whereas established cirrhosis is not [2, 4, 13]. Further, joint pain is not alleviated by iron removal. In a survey of 2851 patients with HH, joint pain was alleviated in 9.2% but worsened in 34.0% after removal of iron overload [14]. Moreover, arthritis can occur during the initial phase of phlebotomy in previously asymptomatic patients with HH [15]. The reasons for this somewhat unexpected effect of phlebotomy on joints is unknown, but joint tissues, including cartilage and synovial membrane, might be sensitive to the mobilization of circulating iron induced by phlebotomy.

To address this issue, we investigated the effect of iron removal on joint homeostasis in patients with HH by measuring the serum levels of the biomarker Coll2-1 and its nitrated form Coll2-1NO₂ (biomarkers of type II collagen degradation), the carboxyl propeptide of type II collagen (CPII, a biomarker of type II collagen synthesis), myeloperoxidase (a biomarker of neutrophil activation), the cartilage oligomeric matrix protein and hyaluronic acid.

Patients and methods

Participants

Patients with a recent diagnosis of genetic haemochromatosis who required phlebotomy therapy were enrolled in this longitudinal, prospective study. Patients with a prior diagnosis of primary OA of the hand, knee or hip according to the ACR criteria or joint inflammatory conditions were excluded to avoid interference with the interpretation of joint biomarkers. Genetic haemochromatosis was diagnosed by homozygosity for the C282Y mutation of the

HFE gene and increased transferrin saturation (>45%) and raised serum ferritin level (>200 µg/l). Analysis of the C282Y mutation involved PCR as described [16].

For each patient, just before the first phlebotomy, data were collected on age, sex, BMI (in kg/m²), ferritin level, transferrin saturation, presence of liver dysfunction [defined as an International Normalized Ratio (INR) of >1.4 or albumin level <35 g/dl or bilirubin concentration of >50 µM]; serum creatinine level and if present, global joint pain related to haemochromatosis [on a visual analogue scale (VAS) from 0 to 100 mm]. Liver biopsy was performed or not depending on an algorithm designed to predict the risk of cirrhosis in C282Y homozygous patients [17].

The Institutional Review Board (IRB) of Paris North Hospitals has reviewed and approved this study (No. IRB00006477). All patients gave their written informed consent to participate in this study.

Phlebotomy therapy

Iron depletion was performed by regular removal of 400–500 ml of blood (corresponding to the removal of 200–250 mg of iron) every week until the serum ferritin concentration dropped <50 µg/l. For each patient, the duration of the venesection therapy and the total volume of removed blood to obtain normal ferritin level were recorded.

Blood sampling

Serum was collected in the morning (between 8:00 a.m. and 12:00 p.m.) during the first phlebotomy and the last phlebotomy that achieved iron depletion. Blood was kept at room temperature for at least 1 h, and after coagulation, samples were centrifuged at 1000g for 10 min. Serum aliquots were stored at –80°C until use.

Immunoassays of biomarkers

Coll2-1 and Coll2-1NO₂. Coll2-1 and Coll2-1NO₂ concentrations were measured by two competitive and specific immunoassays (ELISA) [18]. The Coll2-1 immunoassay measured only the amino acid sequence ¹⁰⁸HRGYPGLDG¹¹⁶ in its linear form, whereas the Coll2-1NO₂ immunoassay quantified, with high specificity and affinity, the nitrated amino acid sequence. The limits of detection were 17 nM for Coll2-1 immunoassay and 25 pM for Coll2-1NO₂ immunoassay. The intra- and inter-assay coefficients of variation (CV) were <10%, and the dilution curves were parallel to the standard curve for both assays. The analytical recoveries were a mean of 104.7 and 121.9% for the Coll2-1 and Coll2-1NO₂ assays, respectively.

Briefly, microplates were coated with 200 µl of streptavidine, 0.5 mg/ml, during 48 h. After washing [buffer: Tris 25 mM, NaCl 50 mM, Tween 20 0.2% (v/v), pH 7.3], microtitre plates were blocked with 400 µl/well of blocking buffer [KH₂PO₄ 1.5 mM, Na₂HPO₄ 8 mM, KCl 2 mM, NaCl 138 mM, BSA 0.5% (v/v), pH 7.2] overnight at 4°C. Coll2-1 and Coll2-1NO₂ were conjugated with biotin as described by Rosenquist *et al.* [19]. After washing, 100 µl

of the biotinylated peptides, Coll2-1 at 2.5 ng/ml or Coll2-1NO₂ at 1.25 ng/ml, were added to each well of streptavidine-coated plates and incubated for 2 h at room temperature. After washing, 50 µl of calibrators (synthetic peptide) or unknown samples, diluted in incubation buffer [10 mM phosphate-buffered saline (PBS), 138 mM NaCl, 7% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.0, for the Coll2-1 immunoassay; and 50 mM Tris, 138 mM NaCl, 7% (w/v) BSA, 0.1% (v/v) Tween 20, pH 8.0, for the Coll2-1NO₂ immunoassay], were added to the wells, then 100 µl D3 antibody (for Coll2-1), diluted 1:40 000, or 100 µl D37 antibody (for Coll2-1NO₂), diluted 1:500 000, and incubated for 1 h at room temperature. The dilutions of the antisera and the secondary antibody were in dilution buffer [10 mM PBS, 138 mM NaCl, 0.2% (w/v) BSA, 0.1% (v/v) Tween 20, pH 7.0, for the Coll2-1 immunoassay; and 50 mM Tris, 138 mM NaCl, 0.2% (v/v) BSA, 0.1% (v/v) Tween 20, pH 8.0, for the Coll2-1NO₂ immunoassay]. After washing, 100 µl of peroxidase-conjugated goat antibodies to rabbit IgG (Biosource, Nivelles, Belgium), diluted 1:5000 in incubation buffer, were incubated for 1 h at room temperature. After washing, 100 µl of freshly prepared enzyme substrate (TMB; Biosource) was added to each well. After 15 min, the reaction was stopped with 100 µl of 4 M H₃PO₄. The colouration was read with the use of a microplate reader (Labsystem, Helsinki, Finland) at 450 nm, corrected for absorbance at 650 nm.

MPO. The MPO in sera was measured by a solid-phase, two-site ELISA as described by the manufacturer (ELIZEN MPO; Zentech SA, Liège, Belgium). Briefly, the assay had sensitivity of 0.4 ng/ml, inter- and intra-assay CV < 15%, accuracy between 89 and 104% and dilution curves parallel to the standard curve.

HA. The HA in sera was measured by an enzyme-linked binding protein assay as described by the manufacturer (Kordia Life Science, Leiden, The Netherlands). Briefly, the performance of the assay was a detection limit equal to 10 ng/ml, inter- and intra-assay CV < 10% and accuracy between 96.4 and 104.9%.

COMP. The COMP in sera was measured by a solid-phase, two-site ELISA as described by the manufacturer (AnaMar, Göteborg, Sweden). Briefly, the assay had a detection limit < 0.1 U/l, inter- and intra-assay CV < 5% and dilution curves parallel to the standard curve.

CPII. The CPII in sera was measured by a semi-quantitative competitive immunoassay as described by the manufacturer (Ibex, Montreal, Canada).

Statistical analysis

Data are mean (s.d.) or median (range) depending on distribution. Comparisons between the levels of biomarkers before and after phlebotomy involved Wilcoxon test. Correlations were tested with the non-parametric Spearman correlation test. A two-sided significance level

was fixed at 5%. All tests involved use of StatView, version 5.0 (SAS, Gary, IN, USA).

Results

Demographic characteristics

A total of 18 successive, newly diagnosed and not treated patients with genetic haemochromatosis (10 males) were included in this study. The clinical characteristics of the patients are summarized in Table 1. All patients were homozygous for the C282Y mutation of the HFE gene and had iron overload. At diagnosis, the serum level of ferritin was 627.5 (133–3276) µg/l and the transferrin saturation was 65% (45–103%). None of the patients had liver dysfunction. A liver biopsy was performed in only one patient and disclosed cirrhosis. In these 18 patients, we needed to remove 12.8 (3.2–33.7) l of blood, corresponding to excess iron of 6.4 (1.6–16.85) g, to reach a ferritin value of 30–50 µg/l. Duration of the phlebotomy treatment was 295 (70–670) days. Baseline ferritin level was correlated with body iron store ($r=0.63$; $P=0.008$). Serum creatinine levels were unchanged after iron excess removal: 55.5 (35–73) vs 56.5 (44–77) µmol/l ($P=0.62$). None of the symptomatic patients took a slow-acting drug for OA during the study period. Painful joints were treated with acetaminophen in six patients, and three patients took NSAIDs occasionally.

Effect of iron depletion on biomarkers

Serum levels of both Coll2-1 and CPII, markers of type II collagen degradation and synthesis, respectively, were significantly increased after iron depletion: 80.1 (55.6–113.5) vs 96.0 (48.8–136.3) nM ($P=0.004$) and 731.4 (374.2–1012.3) vs 812.8 (535.8–1165.6) ng/ml ($P=0.03$), respectively (Table 2; Fig. 1). Levels of other biomarkers were not modified by iron removal (Table 2).

Correlation between iron overload and biomarkers

The severity of iron overload, as assessed by serum ferritin levels at diagnosis, was significantly correlated with serum HA level measured before iron depletion ($r=0.60$; $P=0.01$). Ferritin levels were not correlated with any of the other biomarkers (Table 3).

TABLE 1 Characteristics of the 18 patients with haemochromatosis at entry in the study

HFE genotype, <i>n</i>	C282Y/C282Y, 18
Age, mean (s.d.), years	48 (11)
Sex, male/female, <i>n</i>	10/8
BMI, mean (s.d.), kg/m ²	23.8 (2.4)
Ferritin, µg/l	627.5 (133–3276)
Transferrin saturation, %	65 (45–103)
Creatinine, µmol/l	55.5 (35–73)
Liver dysfunction	None

Data are median (range) unless otherwise indicated.

TABLE 2 Biochemical marker levels before and after iron removal by phlebotomy in patients with genetic haemochromatosis

	Before phlebotomy	After phlebotomy	P-value
Coll2-1, nM	80.1 (55.6–113.5)	96.0 (48.8–136.3)	0.004*
CPII, ng/ml	731.4 (374.2–1012.3)	812.8 (535.8–1165.6)	0.03*
Coll2-1NO ₂ , nM	0.2 (0.1–0.3)	0.2 (0.1–0.2)	0.26
HA, ng/ml	305.4 (84.7–1699.1)	330.8 (121.5–1829.4)	0.14
COMP, U/l	14.7 (7.2–18.9)	12.99 (5.01–19.1)	0.09
MPO, ng/ml	253.9 (46.6–840.2)	251.05 (51.4–563)	0.91

Data are median (range). *Statistically significant.

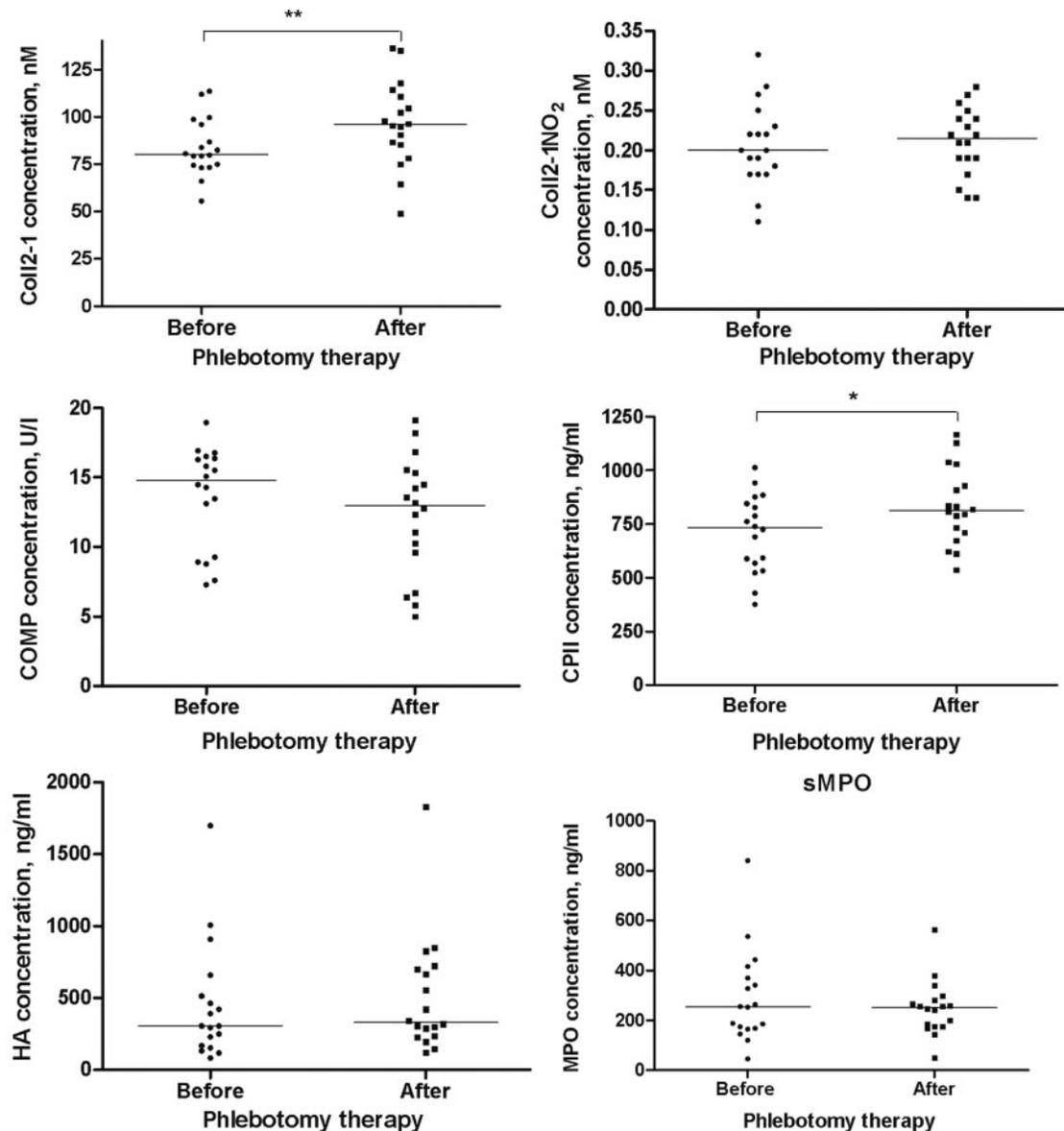
FIG. 1 Individual values of biomarkers before and after phlebotomy therapy in patients with haemochromatosis. The solid lines represent the median value in each group.

TABLE 3 Correlation between ferritin levels at diagnosis and biochemical markers in patients with genetic haemochromatosis

	Spearman test (<i>r</i>)	<i>P</i> -value
Coll2-1	−0.05	0.82
CPII	−0.17	0.48
Coll2-1NO ₂	0.16	0.51
HA	0.6	0.01*
COMP	0.37	0.13
MPO	0.32	0.17

*Statistically significant.

Effect of iron depletion on joint symptoms

Seven of the 18 patients complained of arthralgia of the hands, knees or ankles. Global joint pain in these patients (0–100 mm VAS) before iron removal was 43 (19–73) mm. No patient presented with joint effusion or synovitis. Excess iron removal did not significantly modify joint pain intensity: 36 (16–67) mm ($P=0.07$). No correlations were found between pain intensity and levels of serum ferritin or other biomarkers.

Discussion

Our study provides new insight into the effects of iron removal on cartilage homeostasis. We found an increase in type II collagen turnover following iron depletion in patients with HH, which suggests that cartilage is sensitive to iron mobilization.

The accumulation of excess amounts of iron in body can damage various organ systems, including cartilage. In HH, the potentially toxic form of iron that might be responsible for iron-related damage is labile plasma iron, which represents the redox-active component of non-transferrin-bound iron [20]. Experimentally, iron promotes the formation of free hydroxyl radicals via the Haber–Weiss reaction. Derivative reactive oxygen species are in turn able to induce lipid peroxidation and damage in iron-overloaded tissue [3], including cartilage collagen cleavage [21], and thus could be responsible for the metabolic changes observed in the cartilage.

To investigate the effect of iron removal on cartilage homeostasis, we evaluated type II collagen turnover before the first phlebotomy and after the last phlebotomy to achieve iron depletion. Type II collagen is the most abundant and specific protein of hyaline cartilage. For this reason, numerous type II collagen epitopes have been considered to be the potential biochemical biomarkers of cartilage metabolism. We measured the serum levels of CPII, a marker of type II collagen synthesis, and those of Coll2-1 and its nitrated form, Coll2-1NO₂ [22], which are two of the most recently validated biomarkers of type II collagen degradation [18, 23–25].

CPII is an epitope of propeptide protein fragment. In the process of type II collagen fibril formation, c-propeptide is removed from the procollagen extracellularly and directly

reflects the rate of type II procollagen synthesis [22, 26, 27]. We found a significant increase in the serum concentration of CPII after iron removal, which suggests that type II collagen synthesis was repressed early by iron overload in HH patients. There were no data in the literature on the effect of iron on cartilage collagen synthesis, but in agreement with this result, a previous study [28] has reported that hepatic collagen synthesis might be impaired by iron overload.

Given the important role reactive oxygen species play in the OA cartilage degradation process [29], we hypothesized that iron-induced oxidative stress could be responsible for collagen network degradation. This hypothesis was supported by systemic oxidative stress observed in patients with HH [30]. Surprisingly, following phlebotomy, serum Coll2-1 level, a marker of type II collagen degradation, was increased, whereas those of Coll2-1NO₂ and MPO, two markers of oxidative stress, were not modified. Taken together, these data suggest that oxidative stress, persistent despite iron excess removal [30], is probably not directly involved in the cartilage collagen metabolic changes associated with HH and that iron depletion stimulates type II collagen turnover. Further investigations are needed to explain this result. One possible explanation could be that iron overload could slow down cartilage cell metabolism and progressive iron removal restores normal cell activity.

Interestingly, the effect of iron depletion on joint homeostasis seems to be restricted to type II collagen turnover. Indeed, we found no modification in the level of COMP, an abundant cartilage protein and a member of a thrombospondin family of extracellular proteins [31], nor in the level of HA, considered a marker of synovitis and to a lesser extent cartilage degradation [32, 33].

Our work also demonstrated that phlebotomy failed to relieve joint pain, which agrees with results from previous studies [6, 7, 14]. Of note, innately low levels of hepcidin found in patients with HH are decreased during iron depletion [34, 35] and hepcidin may have anti-inflammatory properties in joints through NF- κ B impairment [36]. This decrease in hepcidin levels following excess removal might explain in part the frequent lack of efficacy of phlebotomy in alleviating joint symptoms, as was found in our study, and the observation of worsened inflammatory joint pain in some patients with HH during the depletion phase of the phlebotomy treatment [14, 37].

We found a strong correlation between ferritin level and the amount of iron removal, which indicates that ferritin concentration at diagnosis can be used as a marker of severity of iron overload. Interestingly, the unique biomarker that significantly correlated with ferritin level was HA, which is overproduced by synovial membrane (SM) cells from arthropathic joints. This suggests that the severity of iron overload is associated with increased production and release of HA from joints in patients with HH and that the synovium is sensitive to iron excess. The latter assessment is also supported by *in vitro* studies showing that iron could stimulate synovial fibroblast proliferation at least in part by down-regulating PGE2 production

[38, 39]. However, this finding should be interpreted cautiously, because the distribution of HA is not limited to articular tissues but, rather, encompasses much of the extracellular matrix. Because of this observation and the systemic metabolism of HA, serum levels of HA can be influenced by conditions other than arthropathy and, in particular, liver dysfunction [40]. In a recent study [40] of patients homozygous for the C282Y mutation, HA was correlated with serum ferritin levels, but this correlation was principally due to the marked increase in HA levels in subjects with liver dysfunction; in contrast, HA level was in the normal range in patients without hepatic involvement and in the early stages of liver fibrosis. We carefully checked the hepatic status of all our patients, and none presented features of liver dysfunction. Although we cannot exclude that HA level was influenced by a possible reduced clearance by sinusoidal endothelial cells [41], we believe that in our patients, HA originated from iron-overloaded joints.

The lack of correlation between the levels of type II collagen biomarkers and the severity of iron overload agrees with data from some clinical studies [7, 42–45] but not all [10, 11], which failed to find a clear association between ferritin levels and severity or extent of haemochromatotic arthropathy. This finding suggests that factors other than solely iron excess may be responsible for the arthropathy associated with HH. For example, mid-region PTH fragments containing the 44–68 region could play a role in the genesis of the arthropathy of HH [11] and modifying genes might influence the joint involvement [7, 9].

Our study is the first to investigate the effect of iron depletion on joint homeostasis as assessed by joint biomarkers. Modification of the clearance of our biomarkers might have affected their serum concentration; however, this is not a likely explanation for the increase in Coll2-1 and CPII level we found in our patients following phlebotomy treatment, because patients' renal function remained stable during the treatment and no patient presented with liver dysfunction. Moreover, levels of the other biomarkers were unchanged after excess iron removal. Despite the small sample size, which may have missed some weak associations, these results are a step forward in the understanding of the arthropathy of hereditary haemochromatosis. Our results show that an increase in type II collagen turnover occurs after iron excess removal, which indicates that cartilage homeostasis is modified by iron depletion.

Rheumatology key messages

- Cartilage is sensitive to iron excess in patients with hereditary haemochromatosis.
- An increase in type II collagen turnover occurs after iron excess removal.

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