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# Arteriosclerosis, Thrombosis, and Vascular Biology



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# Serum Hepcidin and Macrophage Iron Correlate With MCP-1 Release and Vascular Damage in Patients With Metabolic Syndrome Alterations

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**Objective**—Increased body iron stores and hepcidin have been hypothesized to promote atherosclerosis by inducing macrophage iron accumulation and release of cytokines, but direct demonstration in human cells is lacking. The aim of this study was to evaluate the effect of iron on cytokine release in monocytes *ex vivo* and the correlation with vascular damage and to evaluate the relationship among serum levels of hepcidin, cytokines, and vascular damage in patients with metabolic syndrome alterations.

**Methods and Results**—Manipulation of iron status with ferric ammonium citrate and hepcidin-25 induced monocyte chemoattractant protein (MCP)-1 and interleukin-6 in human differentiating monocytes of patients with hyperferritinemia associated with the metabolic syndrome ( $n=11$ ), but not in subjects with hemochromatosis or *HFE* mutations impairing iron accumulation ( $n=15$ ), and the degree of induction correlated with the presence of carotid plaques, detected by echocolor-Doppler. In monocytes of healthy subjects ( $n=7$ ), iron and hepcidin increased the mRNA levels and release of MCP-1, but not of interleukin-6. In 130 patients with metabolic alterations, MCP-1 levels, as detected by ELISA, were correlated with hepcidin-25 measured by time-of-flight mass spectrometry ( $P=0.005$ ) and were an independent predictor of the presence of carotid plaques ( $P=0.05$ ).

**Conclusion**—Hepcidin and macrophage iron correlate with MCP-1 release and vascular damage in high-risk individuals with metabolic alterations. (*Arterioscler Thromb Vasc Biol.* 2011;31:683-690.)

**Key Words:** atherosclerosis ■ cytokines ■ free radicals/free-radical scavengers ■ macrophages ■ risk factors ■ iron

Iron deposition in arterial wall macrophages is increased in atherosclerotic lesions,<sup>1,2</sup> and increased iron stores have been suggested as a marker of cardiovascular risk.<sup>3</sup> Several mechanisms have been proposed to explain the atherogenic role of iron through oxidative stress catalysis, including promotion of insulin resistance,<sup>4</sup> decreased plasma antioxidant activity, and increased low-density lipoprotein (LDL) oxidation,<sup>5</sup> direct endothelial toxicity,<sup>6</sup> enhanced macrophage activation determining oxidized LDL uptake,<sup>7</sup> and accelerated arterial thrombosis.<sup>8</sup> Consistently with the previous findings, iron depletion has been shown to decrease atherogenesis in experimental models,<sup>3,9</sup> blood donation was associated with decreased risk for myocardial infarction,<sup>10</sup> and phlebotomy was suggested to decrease the progression of peripheral vascular disease.<sup>11</sup> However, evidence of the atherogenic effect of iron still controversial.<sup>12,13</sup>

Hepcidin has recently emerged as the key hormone regulating iron balance.<sup>14,15</sup> Hepcidin, whose levels are increased by iron stores and inflammation, inhibits iron recycling from

macrophages and absorption from enterocytes by binding the cellular iron exporter ferroportin, thus matching iron availability to body stores and providing a defense mechanism against extracellular pathogens during inflammation. Increased iron absorption resulting from decreased hepcidin release, related to common missense mutations of the hereditary hemochromatosis (HHC) gene (*HFE*) or other inherited defects, is the common pathogenic mechanism of HHC. Hepcidin may also be induced by subclinical inflammation and obesity,<sup>16</sup> and can determine iron trapping into macrophages,<sup>17</sup> increasing oxidative stress and transformation into foam cells in the presence of an atherogenic environment. Thus, it has been postulated that increased hepcidin may be responsible for iron-induced atherogenesis and may be involved in the mechanism linking inflammation to vascular disease.<sup>3</sup> This theory would explain the controversial association between *HFE* mutations,<sup>18–21</sup> or higher transferrin saturation,<sup>12,13</sup> and susceptibility to vascular disease, which has so far been considered as a negative proof of the “iron hypothesis” of atherosclerosis.

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**Table 1. Clinical Features of Twenty Patients With Nonalcoholic Fatty Liver and MetS Alterations and of Six With C282Y<sup>+/+</sup> HHC, From Whom Peripheral Blood Monocytes Have Been Isolated**

	<i>HFE</i> Status		
	Normal	Iron Overload	HHC
No.	11	9	6
Age, yr	50±9	48±12	49±11
Sex, F	2 (18)	2 (22)	2 (33)
Ferritin, ng/mL*	808±414 (median, 588)	529±310 (median, 546)	708±281 (median, 580)
Transferrin saturation, %*	37±11	36±16	87±10
Metabolic syndrome	9 (82)	7 (78)	1 (17)
Carotid plaques	7 (64)	2 (22)	1 (17)

\*Parameters evaluated at diagnosis; at the time of monocyte collection, all patients were iron depleted and on maintenance venesections. Except where noted otherwise, values in parentheses are percentages.

We recently showed that serum ferritin levels, reflecting iron stores, were an independent predictor of vascular damage in a population of patients with metabolic alterations defining the metabolic syndrome (MetS), but only in those negative for *HFE* genotypes determining decreased release of hepcidin.<sup>3,7,22,23</sup> In addition, recent data confirmed that individuals carrying the major C282Y *HFE* mutation decreasing hepcidin may be at decreased risk for atherosclerosis, whereas vascular damage correlated with iron stores in patients predisposed to iron accumulation.<sup>24</sup> Moreover, we reported an association between hepcidin and carotid vascular damage independently of other risk factors.<sup>22</sup>

However, although oxidative stress leading to increased cytokines release, and especially macrophage chemoattractant protein (MCP)-1, has been suggested to play a role,<sup>25</sup> the mechanism linking increased macrophage iron content with accelerated atherogenesis are far from clear, and there is still no evidence of a modifying effect of *HFE* genotype on the atherogenic effect of iron in macrophages.

The aim of this study was to evaluate the following; (1) the effect of increased intracellular iron content induced by treatment with iron salts and/or hepcidin on the transcription and release of atherogenic cytokines in human differentiating macrophages derived from patients with hyperferritinemia; (2) the modifying effect of *HFE* genotype on cellular iron handling and cytokine release; (3) the correlation between iron induced cytokine release in vitro and vascular damage, and finally; and (4) the correlation between serum levels of hepcidin, proinflammatory mediators, and vascular damage in patients with metabolic alterations with and without hyperferritinemia.

## Methods

### Patients

Experiments in differentiating monocytes were performed on cells isolated from patients on maintenance phlebotomies for nonalcoholic fatty liver disease (NAFLD)/metabolic alterations associated with iron overload,<sup>26</sup> related to the presence or not of *HFE* genotypes predisposing to iron overload, as defined below (n=9 and 11, respectively), and from subjects with HHC (n=6). All patients were iron-depleted and on maintenance phlebotomies at the time of evaluation (Table 1). Additional experiments were conducted in

monocytes of male control subjects with *HFE* wild-type (wt/wt), normal iron parameters, and absence of MetS (n=7).

Vascular damage, iron status, *HFE* genotype, hepcidin-25, and MCP-1 levels were evaluated in 130 consecutive patients with NAFLD with or without hyperferritinemia attending the outpatient service of the Metabolic Liver Disease Center in Milan, for whom serum and DNA samples were available (Table 2).<sup>27</sup>

### *HFE* Genotype and Hepcidin Levels Determination

The C282Y and H63D *HFE* mutations were determined by restriction analysis.<sup>28</sup> We classified *HFE* C282Y/H63D, H63D/H63D, and C282Y/wt as genotypes predisposing to iron overload, whereas *HFE* H63D/wt and wt/wt were considered “normal” genotypes on the

**Table 2. Clinical Features of One Hundred Thirty Patients With Nonalcoholic Fatty Liver Disease and MetS Abnormalities With Complete Characterization of Vascular Damage, Iron Status, Serum Hepcidin-25, and MCP-1 Levels**

Variable	Value	Range
Age, yr	54.4±12	28–77
Sex, F	23 (18)	...
BMI, kg/m <sup>2</sup>	26.7±3.5	19.2–39
Smoke, current or previous	28 (21)	...
Glucose, mmol/L	5.17±0.9	3.46–10.9
HOMA-R	4.2±3.3	3.8–32.7
Diabetes	32 (24.2)	...
Treated hypertension	53 (41)	...
LDL, mmol/L	3.21±0.8	0.72–5.33
HDL, mmol/L	1.31±0.4	0.54–2.46
Triglycerides, mmol/L	1.44±0.7	0.46–5.1
ALT, IU/mL	38.1±24	10–163
Transferrin saturation, %	34.0±11	7–61
Ferritin, ng/mL	399 [175–640]	10–1366
Hyperferritinemia, >320 mol/L/240 F	188 (37.1)	...
Hepcidin, nmol/L	2.2 [0.95–4.25]	0.25–11.7
MCP-1, pg/mL	74.0 [64–157]	1.5–517
Average, CC-IMT mm	0.88±0.22	0.4–1.65
Carotid plaques	44 (34)	...

Values in parentheses are percentages; values in brackets are interquartile range.

basis of a previous study in this series of patients.<sup>29,30</sup> Patients homozygous for the C282Y *HFE* mutation, ie, affected by classic HHC, were used as positive controls of altered macrophage iron handling.<sup>31</sup>

Hepcidin quantification was performed by time-of-flight mass spectrometry as described recently<sup>32</sup> and in serum samples stored at  $-80^{\circ}\text{C}$  before evaluation.<sup>22</sup> MCP-1 quantification was performed in serum samples and cell supernatants by the CCL2/MCP-1 ELISA kit (R&D Systems, Minneapolis, MN).

## Isolation of Monocytes and Gene Expression Analysis

Mononuclear cells were separated using the Lymphoprep method from peripheral blood<sup>33</sup> and treated as described previously.<sup>34</sup> Twenty-four hours after plating, the supernatant was removed leaving differentiating macrophages attached, and new supplemented medium was added in the presence or not of  $150\ \mu\text{mol/L}$  ferric ammonium citrate (FAC), hepcidin-25  $0.5$  or  $3\ \mu\text{mol/L}$  (H-5926; Bachem, Wheel am Rhein, Germany),  $\text{H}_2\text{O}_2$   $10\ \mu\text{mol/L}$ , unless otherwise specified. Cell purity, as confirmed by flow cytometry (with anti-CD14 antibodies), was  $>95\%$  in each case. After further 24 hours cells were harvested and total RNA extracted by the phenol-chloroform method. First-strand cDNA was synthesized with equal amounts ( $1\ \mu\text{g}$ ) of total RNA by the Superscript VILO cDNA synthesis kit (Invitrogen, Carlsbad, CA) with random hexamers. Quantitative real-time PCR analysis of transferrin receptor (TfR)-1, whose expression is decreased with increasing intracellular iron availability,<sup>35</sup> heme oxygenase-1, induced by oxidative stress and involved in neutralizing iron induced oxidative stress,<sup>36</sup> MCP-1, interleukin (IL)-6, tumor necrosis factor (TNF) $\alpha$ , tissue factor, and CD36 mRNA levels, was performed by using the SYBR green chemistry (Fluocycle, Euroclone, Sizzano, Italy). Custom-designed primers and reaction protocols are available on request. All the reactions were performed in triplicate by the Opticon 2 analyzer (Bio-Rad, Hercules, CA) in a  $25\text{-}\mu\text{L}$  final volume. Results were normalized for 18S and  $\beta$ -actin mRNA levels.

## Evaluation of Basal Activation Status of Monocytes

Basal activation status of monocytes was estimated based on the percentage of CD69 positivity of CD14<sup>+</sup> cells at cytofluorimetric analysis.<sup>37</sup> The percentage of monocytes positive for CD69 or CD14 was quantified within 2 hours on a FACScan flow cytometer (Becton Dickinson). Aliquots of around  $100\ \mu\text{L}$  of heparinised blood were submitted to standard triple staining procedures with antiCD14 (F11), antiCD69 (F12) and antiCD45 (F13) monoclonal antibodies (Becton Dickinson, San José, CA).

## Evaluation of Vascular Damage

Vascular damage was determined by measurement of the common carotid arteries intima media thickness (CC-IMT) and plaque detection by echocolor-Doppler ultrasonography, as described previously.<sup>27</sup>

## Statistical Analysis

Data are shown as means $\pm$ SD for normally distributed variables, mean and interquartile range for nonnormally distributed variables (log-transformed before analysis), and frequencies for categorical variables. Data were compared by  $\chi^2$  and Wilcoxon tests, as required, and variables were correlated by Spearman's rho test. Gene expression levels were compared by Wilcoxon signed-rank test for paired samples and by Wilcoxon test for unpaired samples. Logistic regression analysis was performed to adjust the association between MCP-1 and the presence of plaques for confounding variables. Values of  $P\leq 0.05$  were considered statistically significant. All analyses were performed with the JMP 6.0 statistical analysis software (SAS institute, Cary, NJ).

## Results

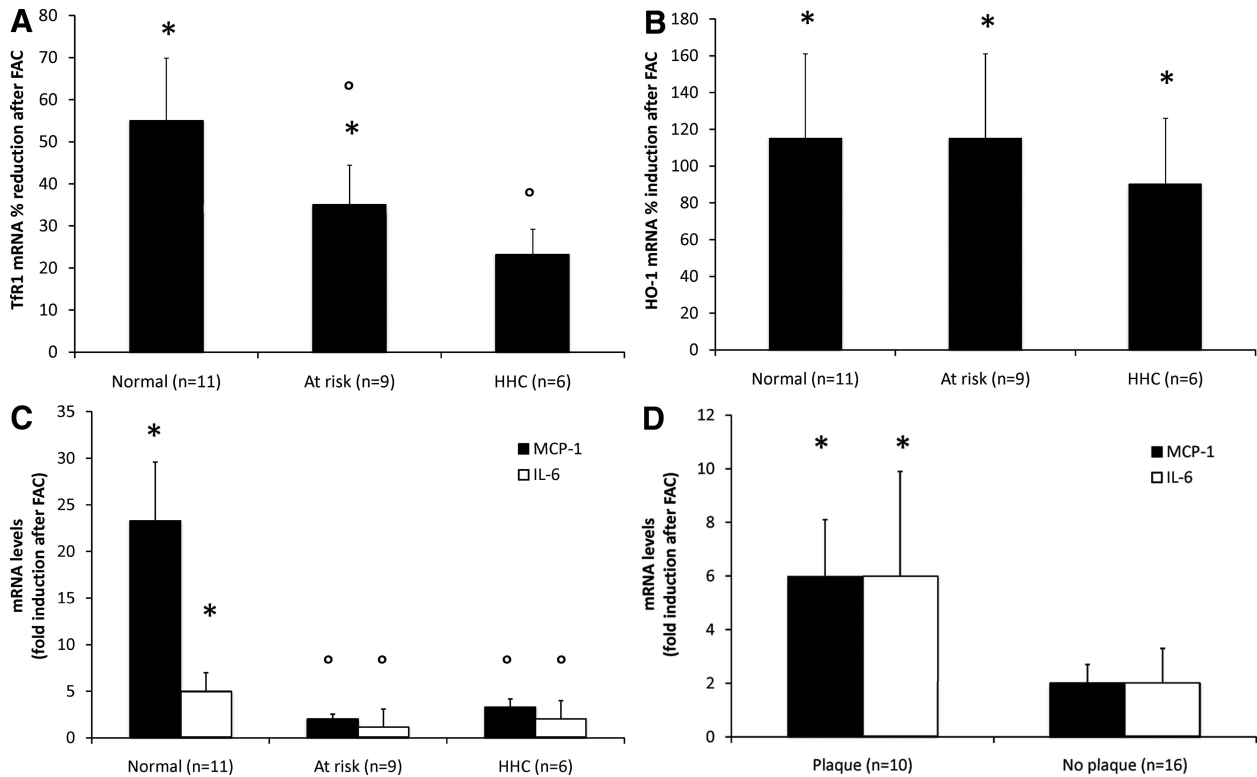
### Risk-Associated HFE Genotypes Influence Iron Handling, But Not Oxidative Stress, in Differentiating Human Monocytes of Patients With Hyperferritinemia

We first confirmed that treatment with iron salts increased intracellular iron status in monocytes of patients with "normal" *HFE* genotype and evaluated whether *HFE* genotypes predisposing to iron overload were associated with altered iron metabolism in macrophages, by comparing the expression of TfR-1 mRNA, a sensitive marker of cellular iron status, in differentiating monocytes isolated from patients with hyperferritinemia related or not to the presence of *HFE* genotypes associated with risk for disease. TfR-1 mRNA transcripts are stabilized during iron deficiency by direct binding of iron regulated proteins to the 3' untranslated regions.<sup>35</sup> Results are shown in Figure 1A. Treatment with FAC determined a significant increase in intracellular iron availability, as detected by a significantly higher decrease in TfR-1 mRNA levels, in monocytes of patients with normal *HFE* genotypes than in those of patients carrying *HFE* genotypes that are associated with risk for iron overload ( $P=0.01$ ), whereas TfR-1 levels were not significantly down-regulated in monocytes of patients with HHC. In contrast, FAC induced a similar upregulation of heme oxygenase-1 mRNA levels irrespective of the *HFE* genotype (Figure 1B). These data suggest that monocytes of patients with *HFE* genotypes associated with risk for iron overload had a partial defect of iron retention reminiscent of that observed in classic HHC,<sup>38</sup> but not reduced oxidative stress.

### Effect of Iron Treatment on the Expression and Release of Atherogenic Factors in Human Differentiating Monocytes is Influenced by HFE Genotype

We next evaluated whether manipulation of iron status modified the release of atherogenic mediators in monocytes of patients with hyperferritinemia subdivided according to the *HFE* genotype (Table 1). Treatment with FAC did not significantly affect either TNF $\alpha$ , tissue factor, or CD36 mRNA levels in monocytes of patients, irrespective of their *HFE* genotype, and basal TNF $\alpha$ , tissue factor, and CD36 mRNA levels were not significantly influenced by *HFE* genotype (data not shown). In contrast, FAC treatment was associated with a significant upregulation of MCP-1 and IL-6 mRNA levels in monocytes of patients with normal *HFE* genotype compared to lack of treatment but not in monocytes of patients with *HFE* genotypes associated with risk for iron overload or with HHC (Figure 1C). Importantly, MCP-1 and IL-6 mRNA levels in untreated cells were not significantly influenced by the *HFE* genotype. Moreover, the percentage of activation (CD69<sup>+</sup>) of peripheral blood monocytes (CD14<sup>+</sup>) was not significantly different in patients with hyperferritinemia subdivided according to the *HFE* genotype (*HFE* normal:  $10.4\pm 15\%$ ,  $n=8$ ; risk *HFE*:  $8.5\pm 14\%$ ,  $n=6$ ; C282Y<sup>+/+</sup> HHC:  $13\pm 15\%$ ,  $n=8$ ; healthy controls with normal ferritin and normal *HFE*:  $7.2\pm 9\%$ ,  $n=6$ ;  $P=\text{NS}$ ). These and previous<sup>39</sup> data suggest that altered *HFE* dysfunction





**Figure 1.** Effect of ferric ammonium citrate (FAC 150  $\mu\text{mol/L}$ ) treatment on Tfr-1 mRNA levels (A), heme oxygenase-1 (HO-1) mRNA levels (B), and mRNA levels (C) of MCP-1 and IL-6 in monocytes of subjects with iron overload subdivided according to *HFE* genotype: “normal,” “hepatic iron overload,” and hereditary hemochromatosis (HHC). \* $P < 0.05$  vs untreated cells;  $^{\circ}P < 0.05$  vs normal *HFE*. D, Association between the induction of MCP-1 and IL-6 mRNA after iron challenge in monocytes of subjects with iron overload and the presence of carotid plaques. \* $P < 0.05$  vs absence of plaques.

does not influence basal activation status but is associated with defective iron retention and a lack of proinflammatory response to iron challenge in human monocytes.

### Inflammatory Response to Iron Challenge in Monocytes Correlates With Vascular Damage in Patients With Hyperferritinemia

To evaluate the relationship between iron-dependent induction of cytokines and vascular damage, we analyzed whether inducibility of MCP-1 and IL-6 in monocytes of patients with hyperferritinemia (Table 1) was associated with the presence of carotid plaques, an index of advanced vascular disease. Results are shown in Figure 1D. Despite basal expression of cytokines not being significantly different in untreated monocytes (data not shown), both MCP-1 and IL-6 mRNA levels were significantly more upregulated after iron challenge in patients with ( $n=10$ ) than in those without ( $n=16$ ) carotid plaques.

### Effect of Iron and Heparin-25 Treatment on Cytokines in Monocytes Derived From Healthy Subjects With Normal Iron Metabolism

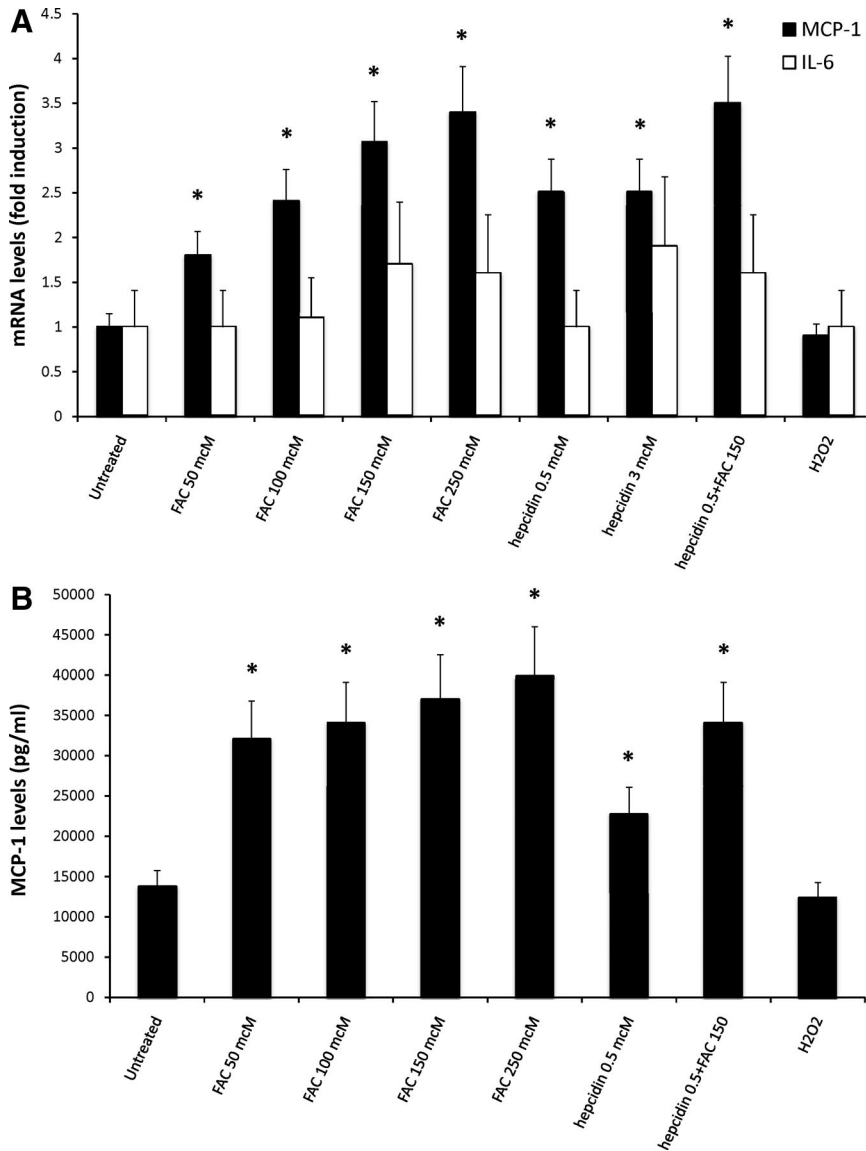
To assess whether MCP-1 and IL-6 induction after iron challenge represented a physiological response to iron overload related to increased iron availability and hepcidin upregulation in monocytes, we next evaluated the dose-response relationship between FAC and hepcidin-25 treatment and MCP-1 and IL-6 mRNA levels in monocytes of 7

healthy male subjects with normal iron metabolism and absence of metabolic alterations. Data are presented in Figure 2A. Surprisingly, neither FAC nor hepcidin-25 induced a significant upregulation of IL-6 mRNA levels in monocytes of most of the healthy subjects ( $n=6/7$ , 86%), whereas increasing concentrations of both FAC and hepcidin-25 induced a progressive increase in MCP-1 mRNA levels. However, we observed a very strong upregulation of IL-6 in 1 healthy control. The absence of MCP-1 upregulation after  $\text{H}_2\text{O}_2$  treatment indicates that the effect of iron is not attributable to an unspecific induction by oxidative stress but is likely a specific response to intracellular iron overload.

Next, we confirmed that increased MCP-1 transcription induced by iron and/or hepcidin-25 treatment translated into increased release of this chemokine in differentiating monocytes, by measuring MCP-1 protein levels in cell supernatants by ELISA (Figure 2B). Data indicate that the maximum MCP-1 induction by FAC was already observed at 24 hours.

### Serum Heparin-25, MCP-1, and Vascular Damage in Patients With Metabolic Alterations

We finally evaluated the relationship among iron status, hepcidin-25, and MCP-1 serum levels and vascular damage in 130 patients with NAFLD and MetS alterations.<sup>27</sup> Heparin-25 levels were not correlated with available demographic and anthropometric features, the presence of diabetes, dyslipidemia, hypertension, central obesity, the metabolic syndrome, and with indices of inflammation or of altered



**Figure 2.** Dose–effect relationship between FAC and hepcidin-25 concentrations in culture medium and the transcriptional induction of MCP-1 and IL-6 in human differentiating monocytes from healthy subjects (A) and the release of MCP-1 in human differentiating monocytes from healthy subjects (B). Monocytes were incubated in the presence of the aforementioned treatments for 24 hours. FAC indicates ferric ammonium citrate. Results represent the means  $\pm$  SD of 2 independent experiments. \* $P < 0.05$  vs untreated cells.

metabolism, but, as previously reported,<sup>22</sup> with serum ferritin, an index of body iron stores ( $Rho=0.43$ ;  $P < 0.0001$ ), and with serum MCP-1 levels ( $Rho=0.24$ ;  $P=0.005$ ; Figure 3A).

In contrast, MCP-1 levels were not significantly correlated with the other available demographic and anthropometric features, and with indices of dysmetabolism and inflammation, including CRP levels ( $\rho=0.03$ ,  $P=0.78$ ). Interestingly, hepcidin levels were significantly correlated with MCP-1 in patients without ( $n=98$ ,  $\rho=0.29$ ,  $P=0.003$ ) but not in those with diabetes ( $n=32$ ,  $\rho=0.12$ ,  $P=0.05$ ), suggesting that in the presence of diabetes, hyperglycemia and its metabolic consequences plays the major role in determining serum MCP-1 levels. However, serum MCP-1 levels were significantly higher in patients with ( $n=44$ ) than in those without ( $n=86$ ) carotid plaques (121 pg/mL [interquartile range, 78 to 193 pg/mL] versus 85 pg/mL [interquartile range, 60 to 148 pg/mL];  $P=0.03$ ; Figure 3B).

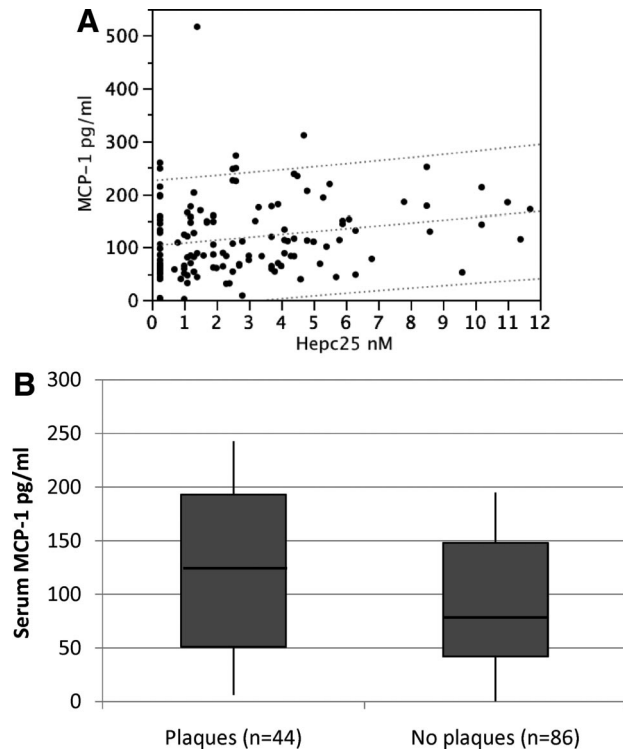
Patients with CRP levels above median value ( $\geq 0.1$  mg/dL) had higher ferritin ( $542 \pm 250$  ng/mL versus  $366 \pm 260$ ;  $P=0.02$ ) and a trend for higher hepcidin-25

( $3.5 \pm 3.2$  versus  $2.5 \pm 1.7$  nmol/L;  $P=0.08$ ) and IMT levels ( $0.92 \pm 0.2$  versus  $0.80 \pm 0.2$ ;  $P=0.08$ ) but not higher MCP-1 levels ( $117 \pm 80$  versus  $113 \pm 60$  pg/mL;  $P=0.7$ ) or prevalence of plaques (59 versus 63%;  $P=0.7$ ). These data suggest that in this series of patients the association between iron indices and MCP-1 levels, and the association between MCP-1 and vascular damage, is independent of subclinical inflammation.

At multivariate analysis (Table 3), presence of carotid plaques was significantly associated with ferritin ( $P=0.013$ ) and MCP-1 ( $P=0.05$ ) serum levels, independently of classic risk factors for atherosclerosis and of the presence of *HFE* mutations.

## Discussion

In this study, we evaluated the effect of the manipulation of intracellular iron status by treatment with iron salts or the hormone hepcidin on the release of atherogenic cytokines in human differentiating monocytes of patients with mild iron overload at high risk for cardiovascular disease.<sup>22,27</sup> The study was prompted by accumulating evidence indicating that



**Figure 3.** A, Correlation between serum hepcidin-25 and MCP-1 levels in 130 patients with NAFLD and MetS abnormalities ( $Rho=0.24$ ;  $P=0.005$ ). B, Comparison of serum MCP-1 levels between patients with and without carotid plaques ( $P=0.03$ ). Median values, interquartile range, and 10th/90th centiles are shown.

iron overload may represent a risk factor for cardiovascular diseases and that the mechanism may be mediated by iron accumulation in arterial plaques macrophages, with consequent induction of inflammation, release of proinflammatory mediators, and transformation into foam cells.<sup>3,22,40</sup> Indeed, infiltration of the arterial wall by activated macrophages that phagocytize oxidative stress–modified lipoproteins and secretion of proinflammatory, fibrogenic, and extracellular matrix remodeling mediators play a pivotal role in the pathogenesis

**Table 3. Independent Predictors of the Presence of Carotid Plaques in One Hundred Thirty Patients With Nonalcoholic Fatty Liver Disease and MetS Alterations as Evaluated by Logistic Regression Analysis**

Variable	OR	95% CI	<i>P</i>
Age, yr	1.12	1.06–1.18	<0.0001
Sex, male	1.98	0.96–4.54	0.08
BMI, kg/m <sup>2</sup>	0.75	0.65–0.84	<0.0001
Diabetes	3.37	1.22–11.9	0.028
Hypertension	1.94	1.10–3.56	0.025
Treated dyslipidemia	2.60	1.13–6.64	0.03
Active smoking	0.94	0.51–1.76	0.85
<i>HFE</i> mutations	0.90	0.53–1.52	0.70
MCP-1, pg/mL	1.01	1.00–1.02	0.05
Ferritin, ng/mL	1.01	1.00–1.02	0.013

BMI, body mass index; OR, odds ratio. All the independent variables considered in the analysis are shown in the table.

of the progression and complications of atherosclerotic lesions.<sup>41</sup>

Our results indicate that treatment with iron salts determining increased intracellular iron increased the release of MCP-1 and IL-6 independently of oxidative stress. Furthermore, the iron-dependent induction of MCP-1 and IL-6 was associated with the severity of vascular damage in these patients, suggesting that macrophage activation by iron may be involved in the pathogenesis of vascular damage progression in these patients also in vivo.

IL-6 is a proinflammatory cytokine involved in the acute phase response, whose serum levels correlate with cardiovascular risk and have been linked to the inflammatory status within atherosclerotic plaques.<sup>42</sup> MCP-1 (also known as CCL2), a chemokine involved in macrophage recruitment at inflammation sites released by macrophages, but also by smooth muscle cells and endothelial cells, plays a crucial role in both the initiation and progression of atherosclerosis, and MCP-1 serum levels reflect the atherosclerotic plaque burden. Higher MCP-1 has also been reported to represent a negative prognostic factor in acute coronary syndromes.<sup>43–45</sup>

A dose-dependent induction of MCP-1 transcription and release in the supernatant induced by iron treatment was confirmed in monocytes of healthy subjects with normal iron parameters, suggesting that this represents a physiological response to increased intracellular iron availability. These data are in line with previous experimental evidence, suggesting that iron may induce MCP-1 release by oxidative stress in macrophages. Indeed, intravenous iron treatment induces MCP-1 release by monocytes in mice and patients with end-stage renal disease,<sup>46,47</sup> whereas the iron chelator deferoxamine has recently been shown to decrease nuclear factor  $\kappa B$  activation, MCP-1 release, macrophage infiltration in vascular tissue, and atherosclerosis progression in ApoE<sup>-/-</sup> mice.<sup>25</sup> In contrast, possibly because of different cellular iron handling caused by the absence of additional acquired and/or genetic factors, we could not confirm an induction of IL-6 by iron treatment in monocytes of most of the healthy subjects.

Importantly, we also showed that treatment with hepcidin, which acts by blocking cellular iron export,<sup>15</sup> mimicked the effect of iron salts on MCP-1 release, as also reported in preliminary form by others.<sup>48</sup> In addition, we obtained evidence that,<sup>38</sup> monocytes of patients with *HFE* genotypes predisposing to mild iron overload show reduced ability to accumulate intracellular iron. Iron-induced upregulation of MCP-1 and IL-6 was hampered in monocytes of patients carrying these disease risk *HFE* genotypes, thus suggesting that the lack of upregulation of proinflammatory mediators may contribute to explain the lack of association between iron overload and accelerated atherosclerosis in HHC patients and explain the contradictory data on the effect of *HFE* mutations on vascular damage.<sup>3,24</sup> Consistently, despite the fact that iron overload has been associated with increased serum MCP-1 levels, patients homozygous for the C282Y *HFE* mutation had lower MCP-1 levels than those whose iron overload was explained by other genetic/acquired factors and healthy controls.<sup>49</sup> Thus, iron-dependent release of MCP-1 by macrophages may be a mechanism involved in the pathogenesis of accelerated atherosclerosis associated with iron overload and



is consistent with the apparently paradoxical protective effect of *HFE* mutations.

To assess whether this mechanism may be relevant in vivo, we measured serum MCP-1 levels in a series of patients at high risk for vascular disease because of MetS and fatty liver, who typically display a high prevalence of altered iron metabolism,<sup>22</sup> with complete characterization of carotid atherosclerosis and iron metabolism, including serum hepcidin-25 evaluation, and *HFE* genotype determination. In line with a possible induction of MCP-1 release by increased iron in monocytes/macrophages, serum MCP-1 levels, whose primary source is represented by activated macrophages, were significantly correlated with hepcidin-25, and MCP-1 levels were an independent predictor of the presence of carotid plaques, indicating an advanced atherosclerotic process.

Thus, the emerging details of the physiology of hepcidin suggest a resolution of the apparent paradox of an important role of iron in atherogenesis in the absence of increased plaque burden in HHC. Hepcidin, induced by iron and inflammation, acts to block iron recycling from macrophages by binding and causing internalization and degradation of ferroportin, the sole cellular iron exporter. Low hepcidin levels are observed in iron deficiency anemia and HHC, both characterized by reduced macrophage iron stores. The failure of vascular wall macrophages to retain iron in HHC may therefore prevent the progression of atherosclerotic plaques.

In conclusion: (1) increased intracellular iron in human differentiating monocytes, induced either by treatment with iron salts or hepcin-25, induces MCP-1 transcription and release; (2) the ability of iron treatment to induce MCP-1 is correlated with the severity of vascular damage; and (3) serum MCP-1 is associated with hepcidin-25 and vascular damage in high-risk individuals with metabolic syndrome alterations. These data support the hypothesis that MCP-1 release by intralésional monocytes/macrophages, which can be iron-loaded because of intraplaque hemorrhage, systemic iron overload, and local inflammation,<sup>3,17</sup> is involved in the pathogenesis of iron-induced atherosclerosis.

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### Disclosures

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