Serum Ferritin Relates to Carotid Intima-Media Thickness in Offspring of Fathers With Higher Serum Ferritin Levels

Anna Prats-Puig,* Maria Moreno,§ Gemma Carreras-Badosa, Judit Bassols, Wifredo Ricart, Abel López-Bermejo, José Manuel Fernández-Real

Objective—Body iron status has been linked to atherosclerosis in adults. The purposes of our study were to determine (1) the association between circulating ferritin levels and carotid intima-media thickness (cIMT) in a cohort of apparently healthy children and (2) the association between cIMT and parental ferritin levels.

Approach and Results—Circulating ferritin levels (microparticle enzyme immunoassay), metabolic parameters, and cIMT (ultrasonography) were analyzed cross-sectionally in a cohort of 692 healthy white children with a mean age of 8±2 years (52% girls and 48% boys). In consecutive 123 children from the cross-sectional sample, the same serum assessments were also performed at baseline in their parents, and the cIMT was repeated after 3 years of follow-up in the children at a mean age of 11±2 years (53% girls and 47% boys). Weak but significant positive associations were evident between children’s circulating ferritin levels and cIMT (r=0.123; P=0.001) and with the change in cIMT 3 years later a tendency was also observed (r=0.185; P=0.048). In multiple regression analyses, circulating ferritin levels contributed independently to cIMT variance (β=0.090; P=0.026; R²=10%) and cIMT change variance (β=0.216; P=0.019; R²= 3.4%) after controlling for body mass index, high-sensitivity C-reactive protein, age, sex, and low-density lipoprotein-cholesterol levels. This association was, however, remarkably significant (β=0.509; P=0.001; R²=20.4%) in children whose fathers had ferritin levels above the median value (122.5 ng/mL). The latter association remained significant after correction for multiple testing. Maternal’s ferritin levels showed no interaction in this association.

Conclusions—These results suggest a paternal-specific effect on cIMT partially reflected by father’s ferritin levels. (Arterioscler Thromb Vasc Biol. 2016;36:174-180. DOI: 10.1161/ATVBAHA.115.306396.)

Key Words: atherosclerosis ■ C-reactive protein ■ carotid intima-media thickness ■ child ■ ferritin

Cardiovascular disease (CVD) is the leading cause of death globally: more people die annually from CVD than from any other cause.1 CVD is extremely rare in children; however, there is a growing interest in the identification of cardiovascular risk factors at an early stage of life because they have long-term effects on vascular health.

Body iron status has been linked to chronic diseases with particular emphasis on atherosclerosis, metabolic syndrome, and diabetes mellitus. Evidences of the iron status on the risk of coronary heart disease were first proposed by Sullivan2 in the 1980s, in the iron hypothesis. This theory defends that overload promotes CVD, whereas, on the contrary, sustained iron deple­tion/deficiency exerts a primary protective effect against ischemic heart disease. Since then, several epidemiological studies have investigated the association between iron stores and the carotid intima-media thickness (cIMT).3–6 In 2007, in his refined iron hypothesis, Sullivan7 proposed that hepcidin might increase CVD risk by trapping iron into macrophages within the plaque, promoting transformation of these cells into foam cells becoming an atherosclerosis promoter.

In an epidemiological study conducted in the general population, Galesloot et al8 demonstrated that serum hepcidin and its ratio to ferritin are associated with atherosclerosis, especially in postmenopausal women. In this line, ferritin was considered an independent predictor of vascular damage in patients with nonalcoholic fatty liver disease, and the mechanism may involve upregulation of hepcidin by increased iron stores.9 This fact was also proved in ex vivo experiments in which treatment with iron salts or hepcidin of human differentiating monocytes induces proinflammatory cytokines release.10 However, the inconsistent effects of iron in atherosclerotic mouse models do not support the hypothesis that iron is an important aggravating factor in the pathogenesis of atherosclerosis.11

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Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>BMI</td>
<td>body mass index</td>
</tr>
<tr>
<td>cIMT</td>
<td>carotid intima-media thickness</td>
</tr>
<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
</tr>
<tr>
<td>hsCRP</td>
<td>high-sensitivity C-reactive protein</td>
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</table>

The relationship between iron stores and cIMT has also been reported in children with end-stage renal disease and with β-thalassemia. It is important to note that these reports have indicated that the association between ferritin and cIMT is also observed in apparently healthy children; however, given the low number of subjects studied (n = 22), these results may only be considered as preliminary.

The intraphenotypic aggregation of flow-mediated vascular dilation has been shown to be significantly higher in father-offspring than in mother-offspring, placing these children at higher risk for CVD. Indeed, the presence of familial metabolic risk factors is known to predict future cardiovascular events in the offspring.

It is well known that increased cIMT is an independent risk marker for coronary artery disease and stroke, becoming a sensitive subclinical atherosclerosis marker. Given the associations found in adults and in children with iron overload, we hypothesized that serum ferritin levels could be linked to cIMT in children from the general population. The purposes of our study were to evaluate (1) the possible association between circulating ferritin levels and cIMT in a cohort of apparently healthy children and (2) the possible relationship between parental ferritin levels and cIMT in the children.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Results for clinical, vascular, and metabolic parameters are shown in Table 1 for all the studied children (n = 692; 333 girls and 359 boys) included in the cross-sectional study and in Table 1 in the online-only Data Supplement for all children included in the longitudinal study (n = 123; 65 girls and 58 boys; Figure 1). Results are shown according to sex. At baseline, boys were slightly older and had higher fasting glucose levels and lower diastolic blood pressure and triglycerides (all P < 0.01).

In the complete sample of children, higher levels of ferritin were associated with higher cIMT values (r = 0.123; P = 0.001) and with the change in cIMT after 3 years of follow-up a tendency was also observed (r = 0.185; P = 0.048; Figure 2; Table II in the online-only Data Supplement). Children’s ferritin levels also showed a tendency with father’s ferritin (r = 0.171; P = 0.015) and were associated with mother’s ferritin levels (r = 0.231; P < 0.0001; Figure II and Table II in the online-only Data Supplement). Notably, father’s ferritin levels were associated with the change in cIMT on follow-up (r = 0.207; P = 0.022; Figure 3; Table II in the online-only Data Supplement) although this association did not remain significant after correcting for multiple testing. Furthermore, in children’s whose fathers had circulating ferritin levels above the median for men (122.5 ng/mL), the association between children’s ferritin and the change in cIMT was remarkably significant (r = 0.520; P < 0.0001; Figure 4). Children’s ferritin levels were also associated with other iron markers such as transferrin (r = 0.202; P < 0.0001) and transferrin saturation (r = 0.207; P < 0.0001), as well as with body mass index (z-score (r = 0.220; P < 0.0001) and high-sensitivity C-reactive protein (hsCRP; r = 0.283; P < 0.0001; Table II in the online-only Data Supplement). However, none of these variables showed significant associations with change in carotid IMT.

In multiple regression analyses, children’s circulating ferritin levels contributed independently to cIMT variance at baseline (β = 0.090; P = 0.026) and the change of cIMT on follow-up (β = 0.216; P = 0.019; R² = 3.4%) after controlling for age, sex, low-density lipoprotein-cholesterol, hsCRP, and body mass index (Table 2). Moreover, in children whose fathers had ferritin levels > 122.5 ng/mL, the associations between children’s ferritin levels and cIMT were remarkably significant (β = 0.509; P = 0.001; R² = 20.4%) in multivariate regression analysis after adjustment for the abovementioned confounding variables: age, sex, low-density lipoprotein-cholesterol, hsCRP, and body mass index (Table 3). The latter association remained significant after correction for multiple testing.

Discussion

The findings of the present study indicate that circulating ferritin levels associate with cIMT and change in cIMT in healthy children; furthermore, the association between children’s ferritin and the change in cIMT was remarkably significant in those children whose fathers have higher ferritin levels.

Body iron stores have been involved in the development of atherosclerotic CVD. In the iron hypothesis, proposed by Sullivan, the effects of iron status on the risk of coronary heart disease were demonstrated. This theory defends that overload promotes CVD, whereas sustained iron depletion/deficiency exerts a protective effect against ischemic heart disease. In 1994, Kiechl et al provided the first information about the relationship between carotid atherosclerosis, sonographically assessed, and ferritin levels. In a cross-sectional study that included 72 healthy men, the plasma-circulating transferrin receptor concentration-plasma ferritin concentration ratio was an independent contributor of cIMT after controlling for traditional risk factors of atherosclerosis. In the Atherosclerosis Risk in Communities (ARIC) study using a matched case–control design, the odds ratio for cases with cIMT versus controls was 1.12 (95% confidence interval, 0.97–1.30). However, there was no association when major cardiovascular risk factors were considered. In this sense, in a cohort of 2443 participants, Wolff et al have reported that serum ferritin levels were not independently associated with cIMT among women or men, despite being associated with carotid plaque prevalence among men.

The underlying mechanism linking iron stores and CVD is not completely understood. However, it has been proposed that increased hepcidin may be responsible for iron-induced athrogenesis. This protein inhibits iron release from enterocytes and macrophages by degrading the iron exporter ferroportin.
Increased hepcidin concentrations might reduce iron mobilization from macrophages with subsequent increased lipid peroxidation and progression to foam cells. This theory is supported by several epidemiological and ex vivo studies performed in adult population. However, the results of a recent article argued against this hypothesis that increased systemic hepcidin promotes macrophage iron retention in atherosclerosis. Liver hepcidin was not influenced by inflammation in a standard mouse model of atherosclerosis (Apoe<sup>−/−</sup>) after 2, 4, or 8 months on high-fat diet. Increased macrophage iron, achieved either through a genetic mutation in the iron exporter ferroportin or through parenteral iron administration, failed

| Table 1. Anthropometrical and Clinical Variables of the Study Subjects (n=692) |
|---------------------------------|-------------------------------|-------------------------------|-----------------|
|                                | All Subjects                  | Girls                        | Boys            |
| n                               | 692                           | 333                          | 359             |
| Age, y                          | 8.1±2.0                       | 7.9±1.9                      | 8.3±2.1         | 0.003 |
| BMI, kg/m²                      | 19.5±4.7                      | 19.2±4.4                     | 19.7±4.9        | ns    |
| BMI Z-score                     | 0.57±1.39                     | 0.57±1.36                    | 0.57±1.42       | ns    |
| Waist:hip ratio                 | 0.79±2.9                      | 0.66±0.26                    | 0.89±0.08       | ns    |
| SBP, mm Hg                      | 105±10                        | 105±10                       | 106±11          | ns    |
| DBP, mm Hg                      | 60±9                          | 61±8                         | 59±9            | 0.008 |
| Carotid IMT, cm                 | 0.040±0.007                   | 0.040±0.007                  | 0.041±0.007     | ns    |
| Fasting glucose, mmol/L         | 86.7±7.3                      | 86.1±6.6                     | 87.4±6.6        | 0.007 |
| HOMA<sub>i</sub>                | 1.2±1.3                       | 1.2±1.2                      | 1.2±1.4         | ns    |
| HDL-cholesterol, mmol/L         | 56.3±13.3                     | 55.7±12.7                    | 56.9±13.8       | ns    |
| Triglycerides, mmol/L           | 63.2±32.6                     | 66.1±33.1                    | 60.5±31.7       | 0.01  |
| LDL-cholesterol                 | 95.0 (29.2–183.2)             | 94.9 (29.2–175.2)            | 95.2 (36.6–183.2)| ns    |
| hsCRP, mg/dL                    | 2.16±3.81                     | 2.26±3.93                    | 2.07±3.70       | ns    |
| Ferritin, ng/mL                 | 40.7 (15.6–65.7)              | 39.0 (28.8–61.0)             | 41.6 (13.6–69.6)| ns    |
| Transferrin                     | 275 (273–278)                 | 266 (261–271)                | 272 (267–278)   | ns    |
| Father’s ferritin, ng/mL        | 144 (130–158)                 | 155 (134–176)                | 132 (113–150)   | ns    |
| Mother’s ferritin, ng/mL        | 26 (23–29)                    | 27 (22–32)                   | 25 (21–29)      | ns    |

Mean±SD or median (interquartile range). BMI indicates body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; hsCRP, high-sensitivity C-reactive protein; IMT, intima-media thickness; LDL, low-density lipoprotein; ns, nonsignificant; and SBP, systolic blood pressure. *P* values are for sex comparison.

Consented to participate at baseline (n=1296)

 Participated at baseline (n=712)

 Withdrawn after consent (n=20)
 Reasons: Data not available (n=20)

 Subjects participating at data collection at baseline (n=692)

 Withdrawn on follow-up (n=596)
 Reasons: Not reached yet time for follow-up (561); did not consent (25); lost on follow-up (10).

Subjects (trios) participating at the follow-up study (n=123)

Figure 1. Flow diagram depicting the inclusion of subjects.
to promote atherosclerosis in mice. Iron might be effectively chaperoned in macrophages by ferritin.\(^{11}\) Notwithstanding, this study could not exclude the possibility that hepcidin is locally increased in macrophages and adipocytes in the plaque environment and may promote macrophage iron accumulation locally.\(^{11}\) On the contrary, Saeed et al\(^{20}\) reported that pharmacological suppression of hepcidin synthesis through inhibition of the BMP (bone morphogenetic protein) pathway decreased atherosclerosis in another mice model. Furthermore, there is a debate whether it is the absolute amount of iron or the distribution of iron in the body that plays a role in the development of atherosclerosis. A small recent study showed that Fe(III) distribution varies substantially within atherosclerotic plaques. Plaques from symptomatic patients had significantly higher concentrations of Fe(III), signs of cap rupture and increased cap macrophage activity.\(^{21}\)

Recent results also suggested that the body iron distribution, as determined by hepcidin, affected the development of atherosclerosis in women. Hecpcidin and the hepcidin/ferritin ratio, reflecting hepcidin expression relative to iron stores, were significantly associated with the presence of plaque in women (adjusted odds ratios for quartile 4 versus quartile 1 [95% confidence intervals], 3.07 [1.36–6.90] and 2.31 [1.03–5.18], respectively). The hepcidin/ferritin ratio was significantly and negatively associated with ankle-brachial index at rest in men and women (adjusted \(β\) for quartile 4 versus quartile 1 [95% confidence intervals], −0.03 [−0.07 to 0.00] and −0.04 [−0.06 to −0.01], respectively).\(^{8}\)

The relationship between iron stores and cIMT was already reported in children with kidney damage\(^{12}\) and with \(β\)-thalassemia.\(^{13–15}\) The mechanism proposed for this connection includes enhanced platelet activation, low-density lipoprotein oxidation, macrophage activity stimulation, and increased nitric oxide destruction in the context of oxidative stress and hemolysis.\(^{22–26}\)

As far as we know, this is the first report showing, in a large sample of healthy white children, that higher circulating ferritin levels are significantly associated with both cIMT and the change in cIMT on follow-up in apparently healthy children. Although the relationship between exposures to biochemical risk factors and IMT was already reported in adults with CVD and children with inherited metabolic diseases,\(^{12–15}\)
data on this relationship in healthy children were lacking. In this sense, data yielded from our study support the association between iron stores and cIMT in a large cohort of healthy prepubertal children, suggesting that circulating ferritin levels may help to identify children at high risk for CVD.

Our results also showed a positive association between children’s ferritin levels and both father’s and mother’s ferritin levels. However, only fathers have an effect on the association between children’s cIMT and children’s ferritin levels. A previous study showed that ferritin, heavy polypeptide-like 17 (FTH117) gene, which encodes a protein involved in iron metabolism, is an imprinted gene predominantly expressed from the paternal allele. Taken together, these results suggest a possible parental-specific effect on cIMT partially reflected by father’s ferritin levels. It may be speculated that higher ferritin levels in fathers may reflect iron accumulation that could influence methylation of genes involved in iron metabolism. The presence of familial metabolic risk factors is known to predict future cardiovascular events in the offspring. However, the intrafamilial aggregation of flow-mediated vascular dilation has been shown to be significantly higher in father-offsprings than in mother-offsprings. It is known that high-density lipoprotein-cholesterol levels are a determinant of flow-mediated vascular dilation and are associated with genetic variation in the Y chromosome. Moreover, it has been postulated that the menstrual cycle could influence

determinant of flow-mediated vascular dilation and are associated with genetic variation in the Y chromosome. Moreover, it has been postulated that the menstrual cycle could influence

Figure 4. Pearson’s correlation for the mean of children’s circulating ferritin levels and children’s increase in carotid intima-media thickness (IMT) on follow-up according to the median of parental ferritin levels in a subsample of 123 trios. ● and ○ depict, respectively, boys and girls.

Table 2. Multivariate Regression Analysis of Carotid IMT (n=692) and Change in Carotid IMT (n=123) as Dependent Variable in the Studied Children

<table>
<thead>
<tr>
<th>Variable</th>
<th>All Children</th>
<th>P Value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotid IMT (n=692)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>0.090</td>
<td>0.026</td>
<td>…</td>
</tr>
<tr>
<td>BMI, km/m²</td>
<td>0.210</td>
<td>&lt;0.0001</td>
<td>…</td>
</tr>
<tr>
<td>hsCRP, mg/dL</td>
<td>0.118</td>
<td>0.013</td>
<td>0.100</td>
</tr>
<tr>
<td>Change in carotid IMT (n=123)†</td>
<td>0.216</td>
<td>0.019</td>
<td>0.034</td>
</tr>
<tr>
<td>Basal ferritin, ng/mL</td>
<td>0.216</td>
<td>0.019</td>
<td>0.034</td>
</tr>
</tbody>
</table>

R² shows the combined effect of the independent variables. BMI indicates body mass index; hsCRP, high-sensitivity C-reactive protein; and IMT, intima-media thickness.

*Nonpredictive variables: age, sex, systolic blood pressure, triglycerides, and low-density lipoprotein-cholesterol.

†Nonpredictive variables: age, sex, body mass index, high-sensitivity C-reactive protein, systolic blood pressure, triglycerides, and low-density lipoprotein-cholesterol.
endothelial function, and this could explain the lack of association between children’s ferritin levels and cIMT according to maternal’s ferritin levels and reinforce the idea of a paternal-specific effect on the association between ferritin levels and cIMT. The relationship between ferritin and hsCRP is of note reinforcing the inflammatory side of serum ferritin concentration. Ferritin is increased in conditions associated with inflammation because, in fact, it is an acute-phase reactant, even in the presence of true iron deficiency. Indeed, it has been considered that hsCRP measurement should be used to adjust for ferritin concentrations. However, we have found that the relationship between cIMT and serum ferritin persists even after adjusting for hsCRP. The lack of association with transferrin saturation could be because of the relatively short range of transferrin saturation in children.

We acknowledge the limitations of our study. The Food and Drug Administration has approved cIMT as a marker of atherosclerosis in adults. However, there is no consensus on noninvasive methods of assessing atherosclerosis in children. Dynamic studies such as direct assessment of endothelial function in children should be performed in future studies. It would be worth evaluating the relationship between iron stores and other atherosclerosis markers such as pulse-wave velocity (a clinical measure of stiffness over an arterial segment) or ankle-brachial index (a surrogate marker for systemic atherosclerosis). Residual confounding by imperfectly or unmeasured variables cannot be excluded. Another possible limitation is that the study population was apparently healthy and needs to be confirmed in, for instance, children with CVD. The relatively small number of subjects included in the longitudinal study warrants further investigation, including replication studies in other populations, to validate current findings. Finally, the facts that the study relies on 1 measurement of ferritin concentration and that HFE (hemochromatosis) gene variant status was not available are also limitation of the current study.

In summary, we herein suggest a paternal-specific effect on the associations between children’s ferritin levels and cIMT because the association is dependent on the sex of the parent. More studies, however, are needed to clarify the nature of this association. Ferritin levels may become a marker of cIMT in healthy children, and in particular, in those children whose father’s have higher levels of ferritin.

Acknowledgments

We are grateful to all the children and parents who took part in the study.

Table 3. Multivariate Regression Analysis of Change in Carotid IMT as Dependent Variable in Asymptomatic Prepubertal Children According to Father’s Ferritin Levels (n=123)

<table>
<thead>
<tr>
<th>Father’s Ferritin &gt;122.5 ng/mL</th>
<th>β</th>
<th>P Value</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in carotid IMT Basal ferritin, ng/mL</td>
<td>0.509</td>
<td>0.001</td>
<td>0.204</td>
</tr>
</tbody>
</table>

R² shows the combined effect of the independent variables. Nonpredictive variables: age, sex, body mass index, high-sensitivity C-reactive protein, systolic blood pressure, triglycerides, and low-density lipoprotein-cholesterol. IMT indicates intima-media thickness.

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Disclosures

None.

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**Significance**

Body iron status has been linked to atherosclerosis in adults; however, studies in children are scanty. We report new evidence that suggest an imprinting effect on carotid intima-media thickness partially reflected by father’s ferritin levels.
Serum Ferritin Relates to Carotid Intima-Media Thickness in Offspring of Fathers With Higher Serum Ferritin Levels

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Materials and Methods

Subjects and Ethics

The study population consisted of 692 school-aged Caucasian children (359 boys and 333 girls, age 8.1 ± 2.0). Children were included in a cross-sectional study of cardiovascular risk factors in prepubertal children from 2007 to 2012. Of these, 123 children (58 boys and 65 girls), whose clinical characteristics did not diverge from the whole group, were longitudinally studied at age ~11 years (after a mean follow-up of 2.9 ± 0.2 yr) and also their parents were studied at baseline, generating a group of family trios (Figure 1). Children were consecutively recruited from well-child visits in primary cares in Girona, a region in Northeastern Spain, and therefore they were all apparently healthy. Participation ranged from 50 to 70% among the different centers.

Inclusion criteria at baseline were: 1) Caucasian origin; 2) age between 5 and 10 years at initial assessment; 3) no pubertal development, as judged by a specifically trained nurse using Tanner criteria (breast stage I; testicular volume < 4 mL). Exclusion criteria were: 1) major congenital anomalies; 2) abnormal blood count, liver or kidney functions 3) evidence of chronic illness or prolonged use of medication; 4) acute illness or use of medications in the month preceding potential enrolment.

The study protocol was approved by the Institutional Review Board of Dr. Josep Trueta Hospital. Informed written consent was obtained from the parents.

Clinical assessments

Clinical examination and venous blood sampling were performed in the morning, in the fasting state. A local anaesthetic cream was used to minimize the discomfort of venipuncture. Weight was measured wearing light clothes with a calibrated scale and height was measured with a Harpenden stadiometer. Body mass index (BMI) was calculated by dividing weight in kilograms by the square of the height in meters. Age- and sex-adjusted Z-score values for BMI were calculated using regional normative data (1). Waist circumference was measured in the supine position at the umbilical level. Hip circumference was
measured at the widest part of the gluteal region. Waist-to-hip ratio was accordingly calculated.

Blood pressure (BP) was measured in the supine position on the right arm after 10 min rest; an electronic sphygmomanometer (Dinamap Pro 100, GE Healthcare, Chalfont St. Giles, United Kingdom) with cuff size appropriate for arm circumference was used. Averages of three readings taken at 5-min intervals were recorded in each subject. None of the children in the current study had hypertension.

**Intima-media thickness**

Carotid IMT was measured by high-resolution ultrasonography (MyLab™ 25, Esaote, Firenze, Italy). For cIMT, diastolic images were obtained using a linear 12-MHz transducer on the right side at the level of the distal common carotid artery, 1 cm away from its bifurcation. Averages of five cIMT measurements on the far wall of the artery were used in the study.

All measurements were taken on a separate visit in all children and were performed by the same observer who was unaware of the clinical and laboratory characteristics of the subjects. Intra-subject coefficient of variation for ultrasound measurements was less than 6%.

**Laboratory variables**

All serum samples were obtained between 8:00 and 9:00 AM under fasting conditions. Serum glucose was analyzed by the hexokinase method. Insulin was measured by immunochemiluminiscence (IMMULITE 2000, Diagnostic Products, Los Angeles, CA). Lower detection limit was 0.4 mIU/L and intra- and inter-assay CVs were less than 10%. Fasting insulin sensitivity was estimated from fasting insulin and glucose levels using the homeostasis model assessment \[\text{HOMA-IR} = \frac{\text{fasting insulin in mU/l}}{405} \times \frac{\text{fasting glucose in mg/dL}}{20}\]. HDL cholesterol was quantified by homogenous method of selective detergent with accelerator. Total serum triglycerides were measured by monitoring the reaction of glycerol-phosphate-oxidase and peroxidase
LDL-cholesterol was estimated by the Friedewald formula (2).

Serum levels of high-sensitivity C-reactive protein (hsCRP) were measured using the ultrasensitive latex immunoassay CRP Vario (Sentinel Diagnostics, Abbott Diagnostics Europe, Milan, Italy). Lower detection limit was 0.2 mg/L and intra- and inter-assay CVs were less than 3%. Children with hsCRP values above 10.0 mg/L were excluded from the study as they indicate the presence of significant acute inflammation (3). Serum ferritin was measured by microparticle enzyme immunoassay (AxSYM; Abbot Laboratories) with intra- and inter-assay CVs < 6%.

**Parental variables**

For each of 123 children, both progenitors were weighed and measured at the initial visit and a blood sample was drawn in order to quantify serum ferritin levels by microparticle enzyme immunoassay (AxSYM; Abbot Laboratories) with intra- and interassay CVs < 6%.

**Statistical analysis**

Statistical analyses were performed using SPSS 19.0 software for Windows (SPSS, Chicago, IL, USA). Normal distribution assumptions were tested using the Kolmogorov-Smirnov test. Variables that were not normally distributed were log10-transformed for further analyses. Results are expressed as means ± standard deviation or median and interquartile range as appropriate for the distribution of variables. Student’s t-test was used to determine differences between genders. The relations between variables were analyzed by simple correlation followed by multiple regression analysis. Statistical significance was set at p < 0.05. Bonferroni correction [conventional p value of 0.05 divided by 8 (number of independent associations analyzed at baseline), yielding a corrected p value ≤0.00625, which is the acceptable level of significance after correcting for multiple comparisons] was also applied to univariate analysis of this study in order to account for multiple testing. The variables included as covariates in the multiple regression where those which are known to be associated with the dependent variable because of physiological reasons plus those which
presented significant Pearson’s correlations with the dependent variable in our study. In our cohort of prepubertal children we estimated a sample size in order to detect a significant Pearson correlation coefficient of at least 0.20 between circulating ferritin and cIMT. Accepting an alpha risk of 0.01, a beta risk of 0.20 and a dropout rate of 0.50, we needed to study 575 children.

References


Supplemental table I: Anthropometric and endocrine-metabolic variables in children included in the prospective study (n=123).

<table>
<thead>
<tr>
<th></th>
<th>All Subjects</th>
<th>Girls</th>
<th>Boys</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>123</td>
<td>65</td>
<td>58</td>
<td>--</td>
</tr>
<tr>
<td>Pubertal children (%)</td>
<td>0.7</td>
<td>1.3</td>
<td>0</td>
<td>ns</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>10.8 ± 1.6</td>
<td>10.9 ± 1.7</td>
<td>10.8 ± 1.6</td>
<td>ns</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>-0.17 ± 1.11</td>
<td>-0.06 ± 1.10</td>
<td>0.14 ± 1.14</td>
<td>ns</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>0.92 ± 0.10</td>
<td>0.92 ± 0.13</td>
<td>0.92 ± 0.1</td>
<td>ns</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>104 ± 11</td>
<td>103 ± 12</td>
<td>104 ± 10</td>
<td>ns</td>
</tr>
<tr>
<td>Carotid IMT (cm)</td>
<td>0.047 ± 0.004</td>
<td>0.047 ± 0.005</td>
<td>0.046 ± 0.004</td>
<td>ns</td>
</tr>
</tbody>
</table>

Abbreviations: BMI, body mass index. Mean ± s.d. or median (interquartile range. p values are for gender comparison. ns: non-significant
Supplemental table II: Correlations between ferritin levels and carotid IMT within the cross-sectional study (N=692).

<table>
<thead>
<tr>
<th></th>
<th>Children’s Ferritin</th>
<th>Change in Carotid IMT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
</tr>
<tr>
<td>Carotid IMT</td>
<td>0.123</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Change in carotid IMT</td>
<td>0.185</td>
<td>0.048</td>
</tr>
<tr>
<td>BMI z-score</td>
<td>0.220</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>hsCRP</td>
<td>0.283</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Transferrin</td>
<td>-0.202</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>-0.207</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>Father’s Ferritin</td>
<td>0.171</td>
<td>0.015</td>
</tr>
<tr>
<td>Mother’s Ferritin</td>
<td>0.231</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
</tbody>
</table>

IMT: intima-media thickness.

P≤0.00625 is considered the acceptable threshold of significance in these data because of the multiple tests performed; these values appeared in bold in the table.