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Title

Low serum iron levels and risk of cardiovascular disease in high risk elderly population: nested case-control study in the PREvención con DIeta MEDiterránea (PREDIMED) trial.

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Summary

Background & aims: Epidemiological data on iron status and cardiovascular disease (CVD) are still controversial. The aim of this study was to determine whether low serum iron (SI) levels are associated with an increased odds of first CVD event in a population at high cardiovascular risk.

Methods: Case-control study design nested within the "PREvención con DIeta MEDiterránea" (PREDIMED) trial. A total of 207 participants diagnosed with CVD (myocardial infarction, stroke or cardiovascular death) during follow-up period (2003-2010) were matched by sex, age and intervention group to 436 controls by incidence density sampling. Median time between serum sample collection and subsequent CVD event occurrence was 0.94 years. Inductively coupled plasma mass spectrometry analysis was used to determine SI levels. In-person interviews, medical

record reviews, and validated questionnaires were used to assess covariates. Multivariable-adjusted odds ratios (ORs) of CVD were calculated with conditional logistic regression.

Results: Mean SI levels were higher in men than in women (1224.0 μg/L vs. 1093.8 μg/L; p<0.001). Among women, but not in men, the mean SI concentration was lower in cases than in controls (1008.5 μg/L vs. 1132.9 μg/L; p=0.030). There was a gradual decrease in the multivariable-adjusted ORs of CVD with increasing SI levels (highest vs. lowest quartile: OR = 0.55, 95% CI: 0.32-0.93; p_{trend} = 0.020). This inverse relationship was more pronounced among women (highest vs. lowest quartile: OR = 0.15, 95% CI: 0.03-0.69; p_{trend} = 0.011)

Conclusions: The present findings are consistent with previously reported inverse associations between SI and CVD. SI levels as an independent marker of short-term cardiovascular risk may be useful for risk assessment in older populations.

Trial registration: www.controlled-trials.com; International Standard Randomized Controlled Trial Number (ISRCTN): 35739639. Registered 5 October 2005. Retrospectively registered.

Keywords: Cardiovascular disease; Serum iron; Older populations; PREDIMED; Prospective studies.

Abbreviations

CHD: Coronary heart disease; CI: Confidence interval; CVD: Cardiovascular disease; HDL: High-density lipoprotein; LDL: Low-density lipoprotein; MedDiet: Mediterranean diet; OR: Odds ratio; PREDIMED: PREvención con DIeta MEDiterránea; SI: Serum iron;

1. Introduction

Iron is an essential trace element with irreplaceable functions in many metabolic processes such as oxygen transport, DNA synthesis, and electron transport. However, an excess of body iron can be harmful due to its redox capability, which may generate reactive oxygen species causing oxidative stress and organic biomolecule oxidation [1]. These processes may contribute to cause cardiovascular disease (CVD) due to the increase formation of atherogenic products such as oxidised low-density lipoproteins (LDL) [2].

During the 1980s Sullivan proposed the iron-heart hypothesis to explain the greater incidence of heart disease in men and postmenopausal women compared to the incidence in premenopausal women, and he suggested that lower iron deposits as a result of menstrual bleeding could explain these differences [3]. The author also linked the worldwide distribution of iron deficiency with lower rates of heart disease. However, despite the physio-pathological mechanism linking body iron stores to CVD, subsequent epidemiological studies have failed to confirm the iron-heart hypothesis and substantial differences have been found depending on the marker used for the assessment of iron status [4–10]. Moreover, contrary to the iron-heart hypothesis, several biomarkers of iron status like serum iron (SI) or transferrin saturation (directly related to SI) have shown robust inverse associations between iron concentrations and CVD [11,12].

Iron may play an important role in the elderly at high cardiovascular risk because physiological and lifestyle factors of ageing, such as changes in absorption-excretion rates, dietary habits or physical activity may affect iron status [13]. Moreover, iron deficiency is the most prevalent comorbidity in patients with heart failure, and this deficiency contributes to a poor prognosis in them [14]. Despite total body iron remains stable through iron homeostasis in the absence of pathologies, among healthy people SI presents short-term variability that makes it an unreliable long-term measure [15]. In addition, SI levels are reduced by factors involved in the pathogenesis of CVD like inflammation and infection [16]. Therefore, SI levels may be a good marker of the short-term risk for CVD in elderly people. Nevertheless, due to the need for sufficient number of events, longitudinal studies

on the relationship between SI levels and CVD have required long periods of follow up, ranged from 4 to 14 years [16–27], and the elapsed times between SI measurement and the occurrence of cardiovascular events are too large to consider SI level as a predictive short-term risk factor of CVD in these studies. A middle/long time frame between SI measurements and cardiovascular events, as considered in previous studies, may be less appropriate for people at high cardiovascular risk due to the possible short-term changes in SI levels associated to CVD pathogenesis. Moreover, due to competing causes of disease and mortality different from CVD events in elderly people, associations based on long-term exposures may be biased. So, it would be interesting to study the relationship between SI levels and subsequent CVD event in elderly individuals at high cardiovascular risk within a shorter time frame.

Although SI levels, among other biomarkers, have been included in a combined score of near-term CVD risk, to the best of our knowledge, the isolated association of SI levels with CVD has not been investigated in elderly people at high cardiovascular risk [25]. Therefore, the aim of the present study was to conduct a prospective nested case-control study within the "PREvención con DIeta MEDiterránea" (PREDIMED) [28,29] study to examine the short-term (< 2 years) associations of SI concentrations with the odds of CVD in a population of Spanish adults aged 55-80 years, at high risk of CVD.

2. Methods and materials

2.1. Study Design

The design of the present study was a paired-matched case-control study nested within the PREDIMED trial (ISRCTN35739639) [29], a multicentre, single-blind, controlled, CVD prevention trial conducted in Spanish primary healthcare centres, aimed at assessing the effects of the Mediterranean diet (MedDiet) on the incidence of CVD. The design and methods of the PREDIMED trial have been described in detail previously [28,30]. Briefly, 7447 community-

125 dwelling men (aged 55-80 years) and women (aged 60-80 years) with no previously documented
126 CVD were recruited. They were considered eligible if they fulfilled at least 1 of 2 criteria: (1) type
127 2 diabetes mellitus or (2) 3 or more of the following cardiovascular risk factors: current smoking,
128 hypertension (blood pressure >140/90 mmHg or treatment with anti-hypertensive drugs), high
129 plasma LDL-cholesterol (>160 mg/dL or treatment with hypolipidemic drugs), low plasma high-
130 density lipoprotein (HDL)-cholesterol (<50 mg/dL in women and <40 mg/dL in men), body mass
131 index ≥25 kg/m^2, or family history of premature coronary heart disease. Exclusion criteria included:
132 history of CVD, any severe chronic illness, or low predicted likelihood of changing dietary habits
133 according to the stages of change model, among others. From October 2003 to June 2009,
134 participants were randomly allocated to a MedDiet supplemented with extra-virgin olive oil, a
135 MedDiet supplemented with mixed nuts, or a control diet consisting of advice to reduce fat intake.
136 The Institutional Review Boards of all the recruitment centres approved the study protocol, and
137 participants provided written informed consent.
138
139 2.2. *Ascertainment of cases and selection of controls*
140 The primary endpoint was a composite of CVD events, defined as myocardial infarction, stroke, or
141 death from cardiovascular causes. Four sources of information were used to identify endpoints:
142 repeated contacts with participants, contacts with family physicians, yearly review of medical
143 records, and consultations of the National Death Index. All medical records related to the endpoints
144 were examined by the endpoint adjudication committee, whose members were blind to intervention
145 allocation. Only endpoints that were confirmed by the adjudication committee and that occurred
146 between October 2003 and December 2010 were included in the analyses.
147 During follow-up, a total of 288 CVD cases were identified. Sixty-two cases had no available serum
148 samples and were excluded from the analysis. For each included case, two controls were selected
149 by incidence density sampling and matched by age at blood collection (±2 years), sex and

intervention group. Participants with serum samples inadequate for mass spectrometry analysis (not enough sample volume or poor sample quality) were excluded (19 cases and 16 controls). Cases not included in the analysis did no differ by lifestyle and baseline characteristics from cases included. The final sample size for the present analysis included 207 cases and 436 matched controls.

2.3. Blood sample collection and measurement of serum iron

Overnight fasting period (≥ 8 hours) blood samples were collected for all participants at baseline and years 1, 3, 5 and 6 (or final visit). Serum samples were coded and processed at each recruiting centre, and aliquots were stored at -80ºC. Laboratory technicians were blinded to the interventions. To assess short-term risk, serum iron was measured by choosing the serum sample closest (preceding) to the event date for each case. Median time between serum sample collection and subsequent CVD event occurrence was 0.94 years (interquartile range (IR): 0.38 – 1.96 years). Among controls, serum samples were chosen with a similar follow-up time of the corresponding matching case (risk set sampling).

For the analysis of SI, a calibration curve was prepared using a 1000 µg/mL Fe standard solution (High-Purity Standards, Charleston, SC, USA) in an solution with 2% (w/v), 1-butanol (Merck, Darmstadt, Germany), 0.05% (w/v), EDTA (Aldrich, St. Louis, MO, USA), 0.05% (w/v) of Triton X-100 (Merck, Darmstadt, Germany) and 1% (w/v) of NH_4OH (Merck, Darmstadt, Germany) in ultrapure water (Milli-Q, Merck, Darmstadt, Germany). Serum samples were diluted 1:10 in the above-mentioned solution. Metal analysis was performed on an Agilent 8900 triple-quadrupole inductively coupled plasma-mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The instrument was tuned, and performance parameters were checked prior to analysis. To ensure the quality of the results, 40 µg/L germanium (ISC Science, Oviedo, Spain) was added to the samples as internal standard. Furthermore, a suitable certified reference material [Seronorm (Sero, Billingstad, Norway) Trace Elements Serum L2 (reference 203105)] was reanalysed together with

175 a blank and an intermediate calibration standard every 12 samples. National Institute of Standards
176 and Technology NIST (USA) Trace Elements in Natural Water Standard Reference Material SRM
177 1640a was also used as certified reference material and analysed at the beginning and at the end of
178 each sequence. Additionally, one in every 12 samples was reanalysed at the end of each session.
179 Repeated re-measurement of a certificate standard serum sample yields an intra-assay coefficient of
180 variation of 5.37% and an inter-assay coefficient of variation of 4.83%. The limit of detection for
181 iron was 8.6 µg/L and there were no concentrations bellow the limit of detection.
182 Participants' glucose, triglyceride, total cholesterol, LDL-cholesterol, and HDL-cholesterol levels
183 were determined locally using fasting plasma samples at baseline. LDL-cholesterol levels were
184 calculated by Friedewald formula whenever triglycerides levels were <300 mg/dL.
185
186 *2.4. Covariate assessment*
187 In a face-to-face interview, trained dietitians completed: (a) a 47-item questionnaire about lifestyle
188 variables, medical history, and medication use. We grouped medications used habitually by
189 participants into 8 categories: angiotensin-converting enzyme inhibitors; diuretics; statins; insulin;
190 aspirin-antiplatelet drugs; calcium channel blockers; angiotensin II receptor antagonists; and beta-
191 blockers; (b) a 14-item validated questionnaire designed to assess adherence to the traditional
192 MedDiet [31]; (c) a validated 137-item semi-quantitative food-frequency questionnaire [32]. For
193 the present analysis, consumption of the following food groups was considered: total meat, red meat
194 and processed meat. Total energy and nutrient intake were calculated on the basis of Spanish food
195 composition tables [33]; and (d) the validated Spanish version of the Minnesota Leisure-Time
196 Physical Activity Questionnaire [34,35]. Trained personnel made anthropometric measurements and
197 measured blood pressure according to the study protocol. The questionnaires were administered and
198 measurements were recorded at baseline and yearly during follow-up visits.
199

2.5. Statistical analyses

Characteristics of cases and controls were described as means and standard deviations for continuous variables and percentages for categorical variables. Group comparisons were carried out using t-test or chi-squared test as appropriate.

SI levels (μg/L) were categorised in sex-specific quartiles with cut-off points based on the distribution among controls [36]. Adjusted levels of covariates across quartiles of SI in controls were estimated using analysis of variance. Polynomial contrast (linear or quadratic trend) were used to evaluate the association of such adjusted levels with quartiles of SI.

To estimate the association between SI concentrations and the odds of incident CVD, we used three conditional logistic regression models (conditional on the matching) with successive degrees of adjustment: (1) with matching factors only, (2) with adjustment for cardiovascular risk factors and potential confounders based on clinical relevance and previous causal knowledge: recruitment centre (indicator variables), current smoker (binary), hypertension (binary), hypercholesterolemia (binary), diabetes (binary), family history of premature coronary heart disease (binary), body mass index (kg/m^2) and alcohol intake (g/day), and (3) with additional adjustment for adherence to MedDiet score (0-14 points), physical activity (METs-min/day), total energy intake (kcal/day), cholesterol intake (mg/day), haem iron intake (mg/day), non-haem iron intake (mg/day), use of angiotensin converting enzyme inhibitors (binary), use of calcium channel blockers (binary), use of angiotensin II receptor antagonists (binary) and use of beta-blockers (binary). Covariates of the last model were included on the basis of the following criteria: (1) dietary iron intake variables (both haem and non-haem) and total energy intake (energy adjustment) [37], (2) variables associated with CVD and/or SI levels at a level of statistical significance $p<0.25$ [38], measured at sample collection time, and without multicollinearity. Sex-specific quartiles of SI was included in the models as categorical variable, and we estimated odds ratios (ORs) and 95% confidence intervals (CIs) for the three upper quartiles using the lowest quartile as the reference category. Tests for trend were

225 performed by assigning each participant the median value of the quartile of SI and treating it as a
226 continuous variable. Similar conditional logistic regression models were fitted for subgroups by sex.
227 We investigated possible interactions between SI and potential effect modifiers by adding a
228 multiplicative interaction term between continuous SI level and the corresponding effect modifier
229 (sex or age) in the models. The statistical significance of interactions was assessed using the
230 likelihood ratio test based on the models with and without the interaction terms.
231 We also used multivariate-adjusted conditional logistic regression to explore the relationship
232 between dietary iron intake and CVD. Moreover, we evaluated the potential non-linear association
233 between SI levels and CVD risk with the use of restricted cubic splines [39].
234 All statistical tests were two-sided, and P values <0.05 were considered statistically significant. All
235 statistical analyses were conducted using Stata 15.1 (Stata Corp).
236
237 **3. Results**
238 *3.1. Characteristics of participants at the time of blood collection*
239 Mean levels of SI were significantly higher in men than in women (1224.0 μg/L vs. 1093.8 μg/L;
240 p<0.001). The main characteristics of the study population at sample collection time are presented
241 in Table 1. Among women, but not men, the mean SI concentration was lower in cases than in
242 controls (1008.5 μg/L vs. 1132.9 μg/L; p=0.030). Cases had a greater proportion of current smokers
243 (20.3% vs. 11.9%; p=0.005) and a higher prevalence of hypercholesterolemia (44.0% vs. 35.3%;
244 p=0.035), hypertension (61.8% vs. 50.9%; p=0.009) and diabetes (62.8% vs. 53.2%; p=0.022) than
245 controls. Concerning dietary intake, we found a greater consumption of red meat in cases than in
246 controls (51.6 g/day vs. 45.2 g/day; p=0.042). The use of statins was significantly less frequent in
247 cases than in controls (25.1% vs. 38.5%; p=0.001), whereas a higher percentage of cases used
248 aspirin-antiplatelet drugs compared to controls (33.8% vs. 22.0%; p=0.001). At baseline, cases had

higher average levels of glucose (137.7 vs. 124.2; p=0.003) and triglycerides (147.6 vs. 130.4; p=0.017) than controls.

3.2. Lifestyle and dietary factors associated with SI in controls

Table 2 summarizes the age-, sex- and centre-adjusted characteristics of controls according to sex-specific quartiles of SI. Among controls, higher alcohol consumption was directly associated with SI levels (p for linear trend=0.011), whereas an inverse association was observed between physical activity and SI (p for linear trend=0.013). The SI concentration was also inversely associated with intake of cholesterol (p for linear trend=0.046), total meat (p for linear trend=0.024), and haem iron (p for linear trend=0.005). Concerning medication use, there was an inverse U-shaped relationship between SI and beta-blockers use (p for quadratic trend=0.041).

3.3. SI and risk of CVD

The associations of SI levels with risk of CVD are presented in Table 3. In the total population, conditional logistic regression models showed that participants in the third and fourth quartiles of SI presented a significantly lower risk of developing CVD than those in the first quartile (OR=0.52, 95% CI: 0.32-0.85 and OR=0.62, 95% CI: 0.40-0.97, respectively; p for trend=0.022). Adjustment for potential confounders increased the association between SI and CVD (OR=0.48, 95% CI: 0.28-0.84 and OR=0.55, 95% CI: 0.32-0.93 for the third and fourth quartile, respectively, vs. the first quartile; p for trend=0.020). P-values for interaction between SI and potential effect modifiers were 0.337 and 0.083 for age and sex, respectively.

To account for a non-linear association, we used restricted cubic spline analysis. The L-shaped association found (Supplementary Figure 1), showed a consistent protection against incident CVD associated with higher levels of SI.

In women, the inverse association between SI and CVD was stronger than that observed in the total population (Table 3). In the fully adjusted model, women in the third and fourth quartiles of SI presented a significantly lower risk of developing CVD than those in the first quartile (OR=0.21, 95% CI: 0.05-0.87, and OR=0.15, 95% CI: 0.03-0.69, respectively; p for trend=0.011).

4. Discussion

In this case-control study within the PREDIMED trial cohort we found that, among Spanish adults aged 55-80 years at high CVD risk, low iron concentrations in serum were associated with increased short-term risk of CVD. These associations were stronger in women.

A total of 13 longitudinal studies have analysed the association between SI and CVD risk, of which 10 studies [16–25] found an inverse association. Two studies [26,27] found no association, and only one study [40] found a direct relationship between high levels of SI (>170 μg/dl) and fatal acute myocardial infarction. In this study, the authors did not find a consistent increase in myocardial infarction risk among participants with SI concentrations in the normal range (considered as 120–174 μg/dl), and several possible sources of bias have been alleged [4]. Our findings concur with the results of previous prospective studies that examined associations of SI levels with CVD incidence in older populations [19–21].

The iron-heart hypothesis was supported by observations such as myocardial failure in iron storage diseases, accumulation of stored iron with age in men, and accumulation of stored iron after menopause to levels found in men. Additional data support this hypothesis [41–43], including the potential involvement of iron in the formation of highly reactive oxygen species and lipid peroxidation, and thereby in the pathogenesis of atherosclerosis and ischemia/reperfusion injury; the protective effect of medication through inhibition of iron absorption, eg, cholestyramine, or gastrointestinal blood loss, eg, aspirin; and the beneficial effects of exercise on CVD by exercise-induced reductions in iron levels. Nevertheless, epidemiological studies have not yielded convincing

298 evidence on the relationship between iron status and CVD, a topic that is still much debated [4–12].
299 Among the reasons for inconsistent results of epidemiological studies (different study populations,
300 diversity of outcomes used, and variable adjustment for confounders), the measurement of iron
301 status yields the greatest discrepancies among studies. For example, a meta-analysis of prospective
302 cohort studies [11] found a borderline positive association between serum ferritin and coronary heart
303 disease (CHD) incidence (relative risk (RR)=1.32, 95% CI: 0.98-1.78), and both SI and transferrin
304 saturation were inversely associated with CHD risk (RR=0.68, 95% CI: 0.58-0.82 and RR=0.76,
305 95% CI: 0.66-0.88, respectively). A possible explanation for these inconsistencies is that the
306 biomarkers used in epidemiological studies may not accurately reflect the real body iron status
307 because they may be influenced by other factors such as inflammation, infection, use of medications,
308 diet, and chronic diseases. We have measured iron status using as a proxy iron concentrations in
309 serum which is a measure of circulating iron bound to transferrin. SI may not be as good a measure
310 of iron storage as serum ferritin, but it is a measure of iron supply to the bone marrow and other
311 tissues, and it has been included among the main biochemical indicators of iron status [44]. More
312 precise measurements of body iron are tissue iron measured by liver biopsy or bone marrow aspirate,
313 but the inconveniences and risks of these techniques limits its use in epidemiological investigations
314 [44,45]. Recent findings [46] have led to the use of genetic-related markers of iron stores in
315 epidemiological studies. Contrary to biomarkers, this approach is not confounded by environmental
316 and lifestyle factors, or reverse causation, and recent mendelian randomization studies suggest a
317 protective effect of higher genetically determined iron status on the risk of CHD [47], some forms
318 of atherosclerotic disease [48] and hypercholesterolemia [49]. Results from these studies, like our
319 findings, do not support the classical iron hypothesis.
320 Iron deficiency is particularly common in the elderly [10,13] and it often causes anaemia. Since
321 anaemia could increase the risk of CVD [50], we have re-analysed our data excluding those
322 participants with SI levels below the normal range defined as SI < 500 μg/L [51]. Eleven participants

(seven cases and four controls) showed SI levels below the normal values and, after excluding them from the analysis, our findings did not materially change (data not shown). Thus, the short-term risk of CVD related to low iron levels observed in our study does not represent the risk associated with iron deficiency, but rather the risk associated with low levels of SI in a population with SI within the normal range.

An interesting observation in our study is a potential sex-specific effect, with a more pronounced CVD risk among women with low SI levels, although the interaction was not statistically significant. Similar to the present study, results from the National Health and Nutrition Examination Survey I (NHANES I) epidemiological follow-up study showed that SI was inversely associated with the risk of CHD in women but not in men [23]. However, three other studies that analysed the association between SI and CVD separately for men and women found that in men, but not in women, low SI was associated with higher risk [17,22,24]. Thus, although our results suggest sex differences in the association between SI levels and CVD, the evidence about the direction of these differences remains unclear and further research is needed for more effective preventive strategies against CVD by sex [52,53].

Our findings about lifestyle and dietary factors associated with SI levels are in agreement with those described in previous studies and enhance the consistency of our results. In our study SI was directly associated with alcohol consumption and inversely associated with physical activity and intake of total meat, cholesterol and haem iron. Since haem iron and meat intake are the main sources of body iron [54], the inverse associations observed between them and SI concentrations suggest that SI not perfectly represents body iron stores. In any case, in our study, there were no associations between dietary iron intake and CVD (Supplementary Table 1).

It is important to interpret the findings of our study in its context because the suggestion that low SI levels may be a risk factor for short-term CVD has potentially significant clinical and public health implications. Our cohort was made of elderly participants at high risk of CVD with normal SI levels.

Medical care in high cardiovascular risk populations may be not as good as expected, as shown, for example, in the lower use of statins in cases despite higher prevalence of hypercholesterolemia (Table 1). These populations are a main target of preventive strategies, therefore the use of SI levels as an independent marker of short-term risk may be useful to reduce primary CVD events in people at high risk. Whether the association between low SI levels and CVD is causal or is a marker of the burden of chronic diseases associated with high CVD risk remains debatable [8]. CVD is a multi-stage disease resulting from several potential pathogenetic pathways that may affect SI levels in a subclinical stage of the disease, when patients are asymptomatic. Thus, low SI levels in an older person should be considered not only as an age-related fact, but also a marker in the pathogenesis of CVD that should alert clinicians. Further studies assessing the relationship between changes in SI concentrations (ideally using repeated-measures) and CVD risk should confirm the clinical usefulness of SI concentrations as a marker of cardiovascular risk.

The causal role of SI in CVD may be supported by some biological evidences. Iron is essential for haemoglobin function and myoglobin synthesis in skeletal and cardiac muscle cells. Iron-containing proteins are necessary for the electron transport system of the respiratory chain to provide energy to the cells, and iron is also required for DNA synthesis and cell proliferation. Finally, iron plays a vital role in the activity of the cardiac conducting system, the brain and the immune system [7,8,19].

The present findings should be interpreted in the context of several limitations. First, the data acquired among a population of high-risk persons living in the Mediterranean region may not be generalizable to other populations. Second, although we have adjusted for many potential confounders, residual confounding cannot be completely ruled out. Inflammation has been associated with decreased SI and transferrin saturation as well as increased serum ferritin [55]. It is also known that several markers of inflammation such as C-reactive protein and fibrinogen are associated with CHD [56]. Thus, inflammation status may be a potential confounder not measured in our study [16]. However, adjustment for inflammatory markers did not substantially change the

373 association observed between iron markers and CVD in several studies [18,20,25,57–59]. Third, the
374 study was conducted in a cohort undergoing a nutritional intervention which might have had some
375 effect on both the incidence of CVD and iron status. We have considered the intervention group as
376 a matching variable, and therefore this effect is minimized. Fourth, although all the comparisons
377 were based on a priori planned contrast, due to the number of comparisons made, we cannot discard
378 the possibility of an increase in global alpha risk due to multiple testing. Fifth, SI is a measure of
379 circulating iron available to tissues and, considering the complexity of body iron homeostasis and
380 the fluctuations of SI concentration, SI levels alone may not accurately reflect the body iron stores.
381 Other biomarkers, such as serum ferritin and transferrin concentrations, are needed for a more
382 accurate assessment of iron status. Nevertheless, we measured iron concentration in serum through
383 inductively coupled plasma mass spectrometry, which is a type of mass spectrometry with extremely
384 high precision and sensitivity, but only capable of measuring metal ions. Therefore, we cannot
385 measure other biochemical indicators of iron status like ferritin and transferrin concentrations.
386 Finally, SI was measured only once, and it may be affected by within-individual variability even
387 though PREDIMED trial was conducted with a well-designed protocol and quality control to
388 minimize such variability [28]. In the absence of widely accepted and validated SI specific cut-off
389 points, we used sex-specific quartiles as an acceptable and unbiased alternative for categorization
390 of the exposure. The single measurement of SI may yield random measurement errors, which tend
391 to attenuate risk estimates. As a result, the inverse association of SI with CVD is likely to be
392 underestimated. Several strengths also deserve comment. The current study was built on a large trial
393 with >4 years of follow up, a well-characterized population, an accurate and blind assessment of
394 incident CVD cases, and controlling for a large number of potential confounding variables. As SI
395 concentration varies diurnally and after meals, blood samples were drawn always in the morning,
396 after a fasting period (≥ 8 hours), and around the same hour. Moreover, parameter estimation and
397 multivariate models were adjusted for centre to take into account possible sample-handling

differences. By using incidence density sampling, we also minimized the possibility of control selection bias. Finally, the short period between SI measurement and event occurrence (<2 years) allows short-term assessment of CVD risk.

5. Conclusions

Contrary to the probably obsolete iron-heart hypothesis, our findings support that high SI levels within the normal range are associated with lower CVD incidence in elderly people at high CVD risk. Although our findings do not necessarily imply that iron supplementation in high risk elderly populations with low SI levels will improve cardiovascular health status, the use of SI levels as an independent marker of short-term risk may be useful in the overall assessment of primary CVD risk in these populations.

Ethics approval and consent to participate

All participants provided written informed consent, and the protocol was approved by the institutional review boards of the participating centres, in accordance with the Declaration of Helsinki. The institutional review board of the Hospital Clínic (Barcelona, Spain), which is accredited by the Department of Health and Human Services and regulated by the Federal wide Assurance for the Protection of Human Subjects of International (Non-US) Institutions (number 00000738), approved the study protocol on July 16, 2002. During enrolment, investigators conducted face-to-face interviews with potential participants, during which the purpose and characteristics of the study were explained.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request and according to PREDIMED confidentiality policies.

Competing interest

The authors declare that they have no competing interests

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449

450 **Authors' contributions**

451 The authors' responsibilities were as follows. MGB and EGG conceived and designed the research;
452 MGB, EGG, MRC and MAMG analysed and interpreted the data; MGB, EGG, MRC and MAMG
453 were responsible for drafting the manuscript. PO and FG conducted the analysis of iron in the serum
454 samples. EGG, MRC, MAMG, JSS, NB, MF, JLdV, DC, JVS, ER, MF, RE, JL, FA, LSM and XP
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649 Table 1. Characteristics of cases and matched controls at sample collection time. The PREDIMED trial.

Characteristic	Case participants	Control participants	p value
n	207	436	
Age (years)	70.9 (6.8)	71.3 (6.6)	Matching Factor
Sex (% women)	37.2	38.5	Matching Factor
PREDIMED trial arm (%)			
Mediterranean diet + EVOO	32.4	31.0	Matching Factor
Mediterranean diet + nuts	27.1	27.5	Matching Factor
Serum iron (μg/L)	1133.2 (420.4)	1193.9 (388.1)	0.072
Men (n = 398)	1207.0 (38.5)	1232.2 (21.4)	0.538
Women (n = 245)	1008.5 (40.6)	1132.9 (33.7)	**0.030**
Smoking status (%)			
Current	20.3	11.9	**0.005**
Former	37.2	34.2	0.453
Hypercholesterolemia (%)	44.0	35.3	**0.035**
Hypertension (%)	61.8	50.9	**0.009**
Type 2 diabetes (%)	62.8	53.2	**0.022**
Family history of CHD (%)	19.8	20.9	0.755
Body mass index (kg/m^2)	29.5 (3.6)	29.2 (3.4)	0.305
Physical activity (METs-min/d)	239.9 (238.4)	275.6 (262.3)	0.097
Alcohol (g/day)	8.8 (15.1)	10.8 (14.6)	0.117
Glucose[a] (mg/dL)	137.7 (52.0)	124.2 (36.9)	**0.003**
Triglycerides[a] (mg/dL)	147.6 (82.6)	130.4 (60.7)	**0.017**
Total cholesterol[a] (mg/dL)	202.4 (33.4)	205.4 (38.9)	0.453
HDL cholesterol[a] (mg/dL)	49.0 (10.1)	50.7 (9.8)	0.107
Mediterranean diet adherence (0 to 14)	9.44 (2.1)	9.8 (2.0)	0.058
Dietary intake			
Total energy intake (kcal/d)	2301.3 (646.2)	2283.3 (560.6)	0.717
Total Fat (%E)	40.1 (6.8)	40.0 (6.6)	0.951
Monounsaturated Fat (%E)	20.3 (4.5)	20.5 (4.4)	0.611
Polyunsaturated Fat (%E)	6.5 (2.1)	6.5 (2.0)	0.889
Saturated Fat (%E)	9.8 (2.3)	9.5 (2.1)	0.096
Carbohydrates (%E)	40.9 (7.3)	40.7 (6.9)	0.680

Cholesterol (mg/d)	373.4 (142.7)	358.1 (129.4)	0.176
Fiber (g/d)	25.3 (9.3)	25.4 (7.7)	0.843
Total Meat (g/day)	132.3 (62.8)	124.8 (51.7)	0.111
Red Meat (g/day)	51.6 (47.2)	45.2 (31.6)	**0.042**
Processed Meat (g/day)	24.7 (18.4)	23.8 (17.8)	0.563
Haem Iron (mg/day)	3.8 (1.6)	3.7 (1.4)	0.410
Non-haem Iron (mg/day)	12.6 (4.1)	12.8 (3.6)	0.495
Educational level (%)			
Primary or less	77.8	79.0	0.941
Secondary	14.0	13.5	
Tertiary	8.2	7.6	
Medication use (%)			
ACE inhibitors	34.8	30.7	0.304
Diuretics	20.3	22.3	0.573
Statins	25.1	38.5	**0.001**
Insulin	7.7	5.5	0.275
Aspirin-antiplatelet drugs	33.8	22.0	**0.001**
Calcium channel blockers	19.3	15.4	0.208
Angiotensin II receptor antagonists	17.4	19.5	0.524
Beta-blockers	13.5	9.9	0.166

650 Data given as mean (standard deviation) or %. EVOO: extra-virgin olive oil; CHD: coronary heart disease; METs:
651 metabolic equivalents; %E: percentage of total energy intake; PREDIMED: PREvención con DIeta MEDiterránea
652 [a]Basal measurement.
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662 Table 2. Adjusted[a] characteristics of 436 controls by sex-specific quartiles of serum iron at sample collection
663 time.

Variables	Quartiles[b] of serum iron				
	Q1	Q2	Q3	Q4	p for linear trend
No. of participants	109	109	109	109	
Median serum iron level (μg/L)					
Men (n = 268)	854.96	1088.57	1305.49	1642.03	NA
Women (n = 178)	674.99	972.23	1187.57	1588.04	NA
Age[c] (years)	71.70	71.76	70.42	71.46	0.465
Body mass index (kg/m^2)	28.53	29.77	29.06	29.62	0.072
Educational level (%)					
Primary or less	76.26	79.66	76.89	82.78	0.333
Tertiary	11.25	3.59	10.06	5.37	0.187
PREDIMED trial arm (%)					
Mediterranean diet + EVOO	29.13	34.03	28.45	32.25	0.850
Mediterranean diet + nuts	22.00	29.31	30.37	28.41	0.295
Smoking status (%)					
Current	10.08	16.66	12.61	8.35	0.496
Former	38.11	27.53	36.38	34.68	0.935
Hypercholesterolemia (%)	31.84	36.47	33.58	39.40	0.316
Hypertension (%)	52.88	50.37	45.30	55.11	0.939
Type 2 diabetes (%)	57.43	56.80	41.64	56.97	0.434
Family history of CHD (%)	27.22	14.44	25.69	16.13	0.199
Physical activity (METs-min/day)	329.56	267.43	267.66	237.88	**0.013**
Alcohol (g/day)	9.04	9.40	11.45	13.24	**0.011**
Glucose[d] (mg/dL)	126.50	123.78	120.78	125.71	0.789
Triglycerides[d] (mg/dL)	129.79	127.84	129.22	134.61	0.634
Total cholesterol[d] (mg/dL)	202.53	209.64	204.91	203.60	0.944
HDL cholesterol[d] (mg/dL)	48.94	51.42	51.31	50.85	0.273
Mediterranean diet adherence (0 to 14)	9.79	9.83	9.82	9.67	0.679
Dietary intake					
Total energy intake (kcal/day)	2280.17	2346.64	2252.14	2254.13	0.465

Total Fat (%E)	39.11	40.98	40.15	39.93	0.548
Monounsaturated Fat (%E)	19.79	20.93	20.49	20.71	0.185
Polyunsaturated Fat (%E)	6.20	6.57	6.66	6.46	0.307
Saturated Fat (%E)	9.36	10.04	9.36	9.26	0.251
Carbohydrates (%E)	41.58	39.84	40.60	40.66	0.473
Cholesterol (mg/day)	358.25	394.20	340.08	339.86	**0.046**
Fiber (g/day)	25.62	25.44	25.04	25.47	0.792
Total Meat (g/day)	127.80	134.10	121.83	115.54	**0.024**
Red Meat (g/day)	42.20	51.04	44.90	42.56	0.701
Processed Meat (g/day)	25.56	24.27	22.81	22.72	0.185
Haem Iron (mg/day)	3.86	4.00	3.57	3.43	**0.005**
Non-haem Iron (mg/day)	12.69	13.00	12.55	13.04	0.707
Medication use (%)					
ACE inhibitors	37.57	33.20	24.20	27.97	0.057
Diuretics	21.58	24.56	22.07	20.78	0.785
Statins	38.86	35.05	38.59	41.62	0.575
Insulin	6.10	8.29	2.91	4.72	0.335
Aspirin-antiplatelet drugs	24.01	23.55	12.93	27.58	0.996
Calcium channel blockers	12.37	11.62	16.74	20.74	0.052
Angiotensin II receptor antagonists	14.08	18.48	22.20	23.23	0.069
Beta-blockers	6.62	10.05	15.55	7.24	**0.041[e]**

Data given as means or %. EVOO: extra-virgin olive oil. CHD: coronary heart disease. METs: metabolic equivalents. %E: percentage of total energy intake. ACE: Angiotensin converting enzyme inhibitors. [a]Adjusted for age, sex and center

[b]Sex-specific quartiles of serum iron based on distribution among controls. Cut-off values for quartiles of serum iron were: 991.29, 1189.39 and 1416.54 μg/L in men and 816.956, 1089.10 and 1324.22 μg/L in women

[c]Adjusted for age and centre

[d]Basal measurement

[e]p for quadratic trend

671 Table 3. Adjusted odds ratios for cardiovascular disease by sex-specific quartiles of serum iron. PREDIMED trial.

		Quartiles[a] of serum iron				
	number of cases/controls	**Q1**	**Q2**	**Q3**	**Q4**	**p for trend**
Total population[b]						
Cases / matched controls	207/436	70/109	54/109	38/109	45/109	
Median serum Fe (μg/L)		783.08	1039.64	1269.33	1618.35	
Matched OR (95% CI)		1 (Ref.)	0.74 (0.47-1.15)	**0.52 (0.32-0.85)**	**0.62 (0.40-0.97**)	**0.022**
Matched OR[c] (95% CI)		1 (Ref.)	0.65 (0.40-1.05)	**0.47 (0.27-0.80)**	**0.57 (0.34-0.93)**	**0.019**
Matched OR[d] (95% CI)		1 (Ref.)	0.62 (0.37-1.03)	**0.48 (0.28-0.84)**	**0.55 (0.32-0.93)**	**0.020**
Men[e]						
Cases / matched controls	130/268	46/67	26/67	25/67	33/67	
Median serum Fe (μg/L)		854.96	1088.57	1305.49	1642.03	
Matched OR (95% CI)		1 (Ref.)	0.56 (0.31-1.01)	0.57 (0.31-1.03)	0.72 (0.42-1.25)	0.304
Matched OR[c] (95% CI)		1 (Ref.)	**0.51 (0.26-0.97)**	**0.48 (0.25-0.95)**	0.62 (0.33-1.15)	0.171

Matched OR[d] (95% CI)		1 (Ref.)	**0.49 (0.25-0.96)**	0.50 (0.25-1.01)	0.69 (0.36-1.33)	0.335
Women[e]						
Cases / matched controls	77/168	24/42	28/42	13/42	12/42	
Median serum Fe (μg/L)		674.99	972.23	1187.57	1558.04	
Matched OR (95% CI)		1 (Ref.)	1.07 (0.53-2.13)	**0.42 (0.18-0.98)**	**0.43 (0.19-0.97**)	**0.017**
Matched OR[c] (95% CI)		1 (Ref.)	0.47 (0.17-1.29)	**0.28 (0.08-0.91)**	**0.35 (0.12-0.99)**	**0.047**
Matched OR[d] (95% CI)		1 (Ref.)	0.46 (0.13-1.59)	**0.21 (0.05-0.87)**	**0.15 (0.03-0.69)**	**0.011**

[a]Sex-specific quartiles of serum iron based on distribution among controls

[b]Models are from conditional logistic regression analyses with matching factors sex, age and intervention group

[c]Adjusted for centre (indicator variables), smoking (binary), hypertension, hypercholesterolemia, diabetes, family history of premature coronary heart disease, body mass index (continuous) and alcohol intake (g/day)

[d]Additionally adjusted for adherence to the Mediterranean diet (continuous), physical activity (continuous), total energy intake (continuous), cholesterol intake (continuous), heme iron intake (continuous), nonheme iron intake (continuous) angiotensin converting enzyme inhibitors use (binary), calcium channel blockers use (binary), angiotensin II receptor antagonists use (binary) and beta-blockers use (binary)

[e]Models are from conditional logistic regression analyses with matching factors age and intervention group.