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Serum iron parameters, *HFE* C282Y genotype, and cognitive performance in older adults: results from the FACIT study

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Running page headline: Iron parameters and cognitive performance

ABSTRACT

Although iron homeostasis is essential for brain functioning, the effects of iron levels on cognitive performance in older individuals have scarcely been investigated. In the present study, serum iron parameters and hemochromatosis (*HFE*) C282Y genotype were determined in 818 older individuals who participated in a 3-year randomized, placebo-controlled, double-blind trial examining the effects of folic acid on carotid intima-media thickness. All

participants had slightly elevated homocysteine levels and were vitamin B12 replete.

Cognitive functioning was assessed at baseline and after 3 years by means of a neuropsychological test battery. At baseline, increased serum ferritin was associated with decreased sensorimotor speed, complex speed, and information processing speed, and increased serum iron was associated with decreased sensorimotor speed. Cognitive performance over 3 years was not associated with *HFE* C282Y genotype or iron parameters. In conclusion, serum iron parameters not show a straightforward relationship with cognitive functioning, although elevated iron levels may decrease cognitive speed in older individuals susceptible to cognitive impairment.

INTRODUCTION

Cognitive function declines with age, even in otherwise healthy individuals. In the last decades, identifying biological determinants of age-related cognitive decline has become an increasingly important goal of aging research (1-3). Disturbed iron homeostasis, which may be genetically or environmentally determined, has been coined as one such suspected risk factor for cognitive decline (4).

Iron deficiency is the most common nutritional deficiency worldwide, with a prevalence of 5-20% among adults in Western countries (5-8). A depletion of body iron stores may result from insufficient dietary intake, hampered absorption, or excessive losses (9, 10). In addition, regular whole blood donation may also lower body iron concentrations (8).

As a constituent of various enzymes, iron is essential for several physiological functions, including oxygen transport and DNA synthesis (11). The brain in particular has a high demand for iron, not only since it is the main oxygen-consuming organ in the human body, but also because many neurobiological processes, including myelination, neurotransmitter synthesis, and synaptic plasticity, are iron-dependent (12-14). Consequently, one of the major symptoms of iron deficiency is reduced cognitive performance (4, 10, 13, 14).

Although the effects of iron deficiency on cognitive functioning have frequently been investigated in children and young adults (15-19), research performed in older persons is rather limited (20-22). Iron deficiency in older individuals may be due to poor dietary habits, gastrointestinal malignancies, or diseases characterized by chronic inflammation (9, 23-25).

Elevated body iron levels might also impair brain functioning. Since body iron concentrations tend to increase with age, particularly in women who reached the menopause (26), increased iron concentrations are more frequent in older persons as compared to young adults; elevated iron levels may be present in up to 20% of community-dwelling individuals

aged 50 years or older (6, 8, 27).

Elevated iron concentrations may also be observed in carriers of the C282Y mutation of the hemochromatosis gene (*HFE*), which causes increased iron absorption (26, 28). The *HFE* C282Y mutation, which has a prevalence of around 10% in communities of Northern European descent (28, 29), has been associated with neurodegenerative disorders (12, 30), including Parkinson's disease (31) and Alzheimer's disease (32, 33).

Despite the fact that both iron deficiency and elevated iron levels frequently occur in older individuals, and are associated with negative effects on cognitive functioning, only a small number of studies have investigated the effects of iron parameters on cognitive performance in later stages of life. The results of these cross-sectional studies are mixed. Whereas Gao and coworkers did not find any associations between plasma iron and cognitive functioning (20), Lam et al. found an inverted U-shaped relationship between serum iron and cognitive functioning in men, and an inverse relationship in women (22). In addition, La Rue and colleagues reported that serum transferrin was positively correlated with cognitive performance in older individuals (21).

The objective of the present study was to examine the cross-sectional and longitudinal associations between iron parameters and cognitive functioning in a large sample of healthy older adults. This study investigated linear and quadratic associations between serum iron parameters and cognitive performance, as well as the relationship between the *HFE* C282Y mutation and cognitive performance.

METHODS

Study population

The study population consisted of 818 men and women who participated in the FACIT study. This randomized, double-blind, placebo-controlled trial was originally designed to investigate the effects of 3-year folic acid supplementation on the risk of cardiovascular disease as measured by carotid intima-media thickness (34). The study sample included a relatively large proportion of blood donors (54%), as participants were recruited from blood bank registries as well as from municipal registries. At screening, individuals were included if between the ages of 50-70 years, and, specifically for women, had reached the menopause at least two years prior. Exclusion criteria were plasma total homocysteine concentrations $<13 \mu\text{mol/L}$ or $>26 \mu\text{mol/L}$, use of B-vitamin supplements or drugs that could affect atherosclerotic progression (e.g. lipid-lowering or hormone replacement therapies), or self-reported intestinal disease. We also excluded individuals with elevated homocysteine concentrations due to factors other than suboptimal folate concentrations, including serum vitamin B12 concentrations $<200 \text{ pmol/L}$, self-reported medical diagnosis of renal or thyroid disorders, or self-reported use of medications that influence folate metabolism. The Medical Ethics Committee at Wageningen University approved the study and all participants gave written informed consent.

Cognitive functioning

Cognitive functioning on the domains of memory, sensorimotor speed, complex speed, information processing speed, and word fluency was assessed at baseline and at 3-year follow-up by means of a neuropsychological test battery, consisting of the Visual Verbal Word Learning Task (35), the Stroop Color-Word Interference Test (36), the Concept Shifting Test (37), the Letter-Digit Substitution Test (38), and the Verbal Fluency Test (39).

A detailed description of the cognitive test battery and the method used for creating cognitive performance indices based on the raw test scores can be found elsewhere (34).

Blood measurements

The iron parameters measured were total serum iron; total iron binding capacity, which is a measure of the serum concentration of the iron transport protein transferrin (40, 41); transferrin saturation, which is expressed as the ratio ($\times 100\%$) of serum iron concentration and total iron binding capacity; serum ferritin, an indicator of total body iron stores (42); and non-transferrin bound iron (43, 44).

At baseline and at 3-year follow-up, fasting venous blood samples were collected, centrifuged within 2 hours, and the serum supernatant was stored in multiple aliquots at -80°C . Within 15 months of storage samples were thawed for serum measurements. Serum iron and total iron binding capacity were measured using Hitachi 747 (Roche Diagnostics). Serum ferritin was determined on the Immulite 1 of DPC (Diagnostic Product Corporation) using a two-site immunometric assay (reference values: 15-190 $\mu\text{g/L}$ for postmenopausal women and 15-280 $\mu\text{g/L}$ for men). Non-transferrin bound iron, which was measured at baseline only, was determined by a fluorescence-based one step chelation method (45). Serum high-sensitivity C-reactive protein was determined at baseline with ELISA using polyclonal antibodies (Dako). An automated hematology analyzer (Sysmex) was used to measure serum hemoglobin. For active blood donors, blood samples were collected at least six weeks after the most recent blood donation.

Genotyping

Genomic DNA was extracted from whole blood samples using a Qiamp 96 DNA blood kit (Qiagen, Venlo, The Netherlands). DNA samples were stored at -80°C until further

analysis. The *HFE* C282Y genotype was determined by an automated method using minor-groove binding DNA oligonucleotides (MGB-probes) (46). The presence of a C282Y allele was confirmed by conventional polymerase chain reaction (PCR) with restriction fragment length polymorphism analysis (47, 48). Apolipoprotein E (*APOE*) genotype was determined as described elsewhere (34).

Other measurements

Level of education, measured at baseline by classifying formal schooling according to the Dutch educational system, was categorized into low/middle/high, i.e. corresponding to primary education, junior vocational training, and senior vocational/academic training, respectively (49). Alcohol consumption (g/day) and current smoking (yes/no) were ascertained at baseline by means of self-report questionnaires, which were reviewed by a trained research assistant. Body mass index (BMI, kg/m²) was calculated from height and weight, and physical activity was estimated using the Physical Activity Scale for the Elderly (50).

Statistical analysis

Cross-sectional analyses

The cross-sectional associations between iron parameters and cognitive functioning were assessed by means of hierarchical linear regression analysis. The iron parameters considered relevant in relation to cognitive performance were total serum iron, ferritin, and non-transferrin bound iron. These parameters represent the different sources of iron in the blood, i.e. total circulating iron, a reflection of total stored body iron, and circulating iron not bound to the plasma transport protein transferrin, respectively (42-44). Transferrin saturation and total iron binding capacity were not included in the regression analyses, not only because

they are indirect measures of body iron levels, but also because including these variables in the statistical models would have introduced multicollinearity.

Separate regression models were fitted for the five cognitive performance indices. The covariates age, sex, level of education, alcohol consumption, smoking, BMI, physical activity, C-reactive protein, hemoglobin, and *APOE* E4 carrier status were entered in step 1, followed by the iron parameters in step 2. Similar regression analyses were performed to examine the associations between the *HFE* C282Y mutation and cognitive performance. The variables age, level of education, alcohol consumption, smoking, BMI, and physical activity were confounders in our study. Although sex, C-reactive protein, hemoglobin, and *APOE* E4 carrier status were no actual confounders in our analyses, we included these variables as covariates to enable comparison with other studies investigating the associations between body iron levels and cognitive performance (6, 22, 51-53). We also tested for a possible confounding effect of homocysteine, as all participants had slightly elevated plasma total homocysteine levels. However, the results were similar regardless if homocysteine was included in the analyses. Therefore, we did not include this variable in the final statistical models.

To examine the possible non-linear relationships between iron parameters and cognitive performance, regression models were fitted for each cognitive performance index, with quadratic terms for iron parameters as the independent variables, adjusting for covariates and linear terms for iron parameters in step 1.

Longitudinal analyses

First, we tested whether treatment with folic acid was an effect modifier in our dataset, as folic acid was found to exert a positive effect on cognitive performance (34), and previous research suggested that folate might interact with iron metabolism (54). We found that folic

acid supplementation was not an effect modifier in our dataset; the interaction terms for iron parameters and treatment condition were not statistically significant (Supplementary Table 1). Furthermore, the serum iron parameters did not significantly differ between the placebo group and the treatment group at the end of the study ($p=0.972$ for serum iron and $p=0.892$ for ferritin), indicating that folic acid supplementation did not influence body iron levels in our study. Therefore, the longitudinal analyses were performed in the total sample.

Using linear mixed models we investigated the associations between iron parameters and cognitive performance over 3 years of follow-up, adjusting for covariates. This analysis method takes into account the correlation between repeated measurements and allows the inclusion of participants with missing observations at follow-up (55). Separate models were fitted for each iron parameter in relation to each of the five cognitive performance indices. An unstructured covariance structure was used. Time (measured in years since baseline) was included to estimate the change in cognitive performance over 3 years of follow-up. The longitudinal effect of the iron parameters was estimated by the two-way interaction between time and the specific iron parameter, which represents the rate of change in cognitive performance over 3 years as a function of this iron parameter. The longitudinal associations between the *HFE* C282Y mutation and cognitive performance were examined in a similar manner.

In secondary analyses, we stratified the study population by sex, donor status, and *APOE* E4 carrier status to determine whether the cross-sectional and longitudinal associations between iron parameters on the one hand and cognitive functioning on the other differed between men and women, blood donors and non-donors, or carriers and non-carriers of the *APOE* E4 allele. One-sample *t* tests were used to determine whether serum iron, serum ferritin, and cognitive functioning changed over the 3-year follow-up period. Chi-square tests and independent samples *t* tests were used to investigate whether the iron parameters serum

iron, total iron binding capacity, and non-transferrin bound iron, as well as the background variables age, sex, level of education, alcohol consumption, smoking, BMI, physical activity, C-reactive protein, hemoglobin, and *APOE* E4 status differed between carriers and non-carriers of the *HFE* C282Y mutation. Because of heterogeneity of error variances, the non-parametric Mann-Whitney U test was performed to test for differences in serum ferritin and transferrin saturation between carriers and non-carriers of the *HFE* C282Y mutation. Hardy-Weinberg equilibrium was assessed using a Chi-square test. Normality of the standardized residuals of the regression analyses was ascertained by means of normal P-P plots.

The statistical power of the cross-sectional and longitudinal analyses was >0.90 (small effect size, $f^2=0.02$). Statistical differences were considered significant at p -values <0.05 . All analyses were performed using SPSS 16.0 for Apple Macintosh (SPSS Inc., Chicago, IL).

RESULTS

Participants

Table 1 shows the baseline characteristics of the participants. Forty-three individuals (5.3%) showed depleted iron stores, as indicated by serum ferritin concentrations below the lower normal limit (i.e. 15 $\mu\text{g/L}$). Thirty-two individuals (3.9%) showed serum hemoglobin concentrations characteristic of anemia according to WHO criteria, i.e. <7.5 mmol/L for women and <8.1 mmol/L for men. The combination of both low ferritin and low hemoglobin levels, i.e. iron deficiency anemia, was present in only seven participants (0.9%). In total, fifty-three participants (6.5%) showed serum ferritin concentrations above the upper normal limit (i.e. 190 $\mu\text{g/L}$ for women and 280 $\mu\text{g/L}$ for men). Among non-donors, the prevalence of low serum ferritin was 0.8%, and the prevalence of high serum ferritin was 13.1%, and among blood donors, these percentages were 11.3% and 0.9%, respectively.

Eighty individuals were identified as carriers of the *HFE* C282Y mutation (10.5%); two individuals were homozygous (0.3%) and 78 persons were heterozygous (10.2%). The allele frequencies of the *HFE* C282Y polymorphism did not significantly differ from the expected distribution predicted by the Hardy-Weinberg equilibrium ($p=0.860$) and were comparable to the frequencies reported in other population-based studies (28, 29). Serum iron, transferrin saturation, and non-transferrin bound iron were significantly increased in carriers of the *HFE* C282Y mutation as compared with non-carriers, whereas total iron binding capacity was decreased in carriers of the *HFE* C282Y mutation as compared with non-carriers (Table 1). Serum ferritin and hemoglobin concentrations, as well as the background variables, did not significantly differ between carriers and non-carriers of the *HFE* C282Y mutation (Table 1). Table 2 presents the serum iron parameters in carriers and non-carriers of the *HFE* C282Y mutation, stratified by *APOE* E4 carrier status.

Cross-sectional associations between serum iron parameters and cognitive functioning

The regression analyses including quadratic terms for the iron parameters did not reveal any curvilinear relationships between iron parameters and cognitive functioning (data not shown). Cross-sectional linear regression analyses indicated that higher serum ferritin levels were significantly associated with decreased sensorimotor speed, complex speed, and information processing speed, after adjustment for age, sex, level of education, alcohol consumption, smoking, BMI, physical activity, C-reactive protein, hemoglobin, and *APOE* E4 carrier status (Table 3). In addition, higher serum iron was associated with decreased sensorimotor speed (Table 3). In order to better understand the strength of the associations, when the predictive value of serum iron for cognitive performance is compared to the relationship with age in the same regression model, an increase of 10 $\mu\text{mol/L}$ serum iron corresponds to a lower performance on sensorimotor speed similar to an individual 5.9 years older. Likewise, a 100 $\mu\text{g/L}$ increase in serum ferritin corresponds to the sensorimotor speed and information processing speed of someone 1.2 years older and the complex speed of an individual 1.3 years older. In contrast to serum iron and serum ferritin, non-transferrin bound iron was not significantly associated with cognitive performance.

The observed associations between iron parameters and cognitive performance did not differ between men and women, except for the relationship between ferritin and sensorimotor speed, which was significant in men ($\beta=-0.089$, $p=0.029$), but not in women ($\beta=0.048$, $p=0.498$). When the analyses were stratified by donor status, only non-donors showed a negative association between serum iron and sensorimotor speed ($\beta=-0.313$, $p=0.003$, as compared to $\beta=-0.093$, $p=0.409$ in donors), which was stronger than the association found in the total sample. Statistical significance of the other associations between iron parameters and cognitive performance that were found in the total sample was eliminated upon stratification by donor status. Stratification by *APOE* E4 carrier status showed that the observed

relationship between ferritin and sensorimotor speed was significant only in carriers of one or two *APOE* E4 alleles ($n=80$) ($\beta=-0.150$, $p=0.023$, as compared to $\beta=-0.044$, $p=0.287$ in the non-carrier group); in comparison with the total sample, the negative relationship between ferritin and sensorimotor speed was more pronounced in *APOE* E4 carriers. In *APOE* E4 carriers, the negative association between serum iron and sensorimotor speed was similar to the relationship observed in the total sample, although it was not statistically significant. Overall, the cross-sectional associations between iron parameters and cognitive performance in the analyses stratified by sex, donor status, or *APOE* E4 carrier status pointed in the same direction as the associations observed in the total sample, with few differences between the groups, although they tended not to reach statistical significance.

In post-hoc analyses, we stratified by serum C-reactive protein concentration (normal ≤ 3 mg/dL, elevated >3 mg/dL (56)) to ascertain whether serum ferritin was associated with cognitive functioning in the absence of inflammation. Higher serum ferritin predicted slower sensorimotor speed ($p=0.008$), complex speed ($p=0.007$), and information processing speed ($p=0.018$) in individuals with normal C-reactive protein concentrations ($n=659$), but not in persons with elevated C-reactive protein levels indicative of inflammation ($n=144$) ($p=0.574$, $p=0.777$, and $p=0.945$, respectively).

Longitudinal associations between serum iron parameters and cognitive functioning

Cognitive performance significantly declined over the 3-year follow-up period on the domains of sensorimotor speed (mean change [95% CI] = -0.08 [-0.11 ; -0.05], $p=0.000$), complex speed (mean change [95% CI] = -0.06 [-0.10 ; -0.02], $p=0.004$), and information processing speed (mean change [95% CI] = -0.11 [-0.15 ; -0.08], $p=0.000$), whereas memory improved significantly (mean change [95% CI] = 0.39 [0.34 ; 0.44], $p=0.000$) due to the effect of procedural learning. Word fluency did not significantly change over 3 years (mean change

[95% CI] = 0.03 [-0.03; 0.09], $p=0.320$). Ferritin concentrations increased significantly over the 3-year follow-up period (mean change [95% CI] = 16.6 $\mu\text{g/L}$ [6.7; 26.4], $p=0.001$), whereas serum iron showed a non-significant decrease (mean change [95% CI] = -0.5 $\mu\text{mol/L}$ [-1.0; 0.01], $p=0.055$). Three-year changes in serum iron and ferritin did not differ between the two experimental groups ($p=0.268$ for serum iron and $p=0.707$ for ferritin), or between men and women ($p=0.637$ for serum iron and $p=0.480$ for ferritin). In addition, the longitudinal changes in serum iron and ferritin did not significantly differ between blood donors and non-donors ($p=0.965$ for serum iron and $p=0.088$ for ferritin), although blood donors showed a larger 3-year increase in ferritin than non-donors (mean change [95% CI] = 24.4 $\mu\text{g/L}$ [8.1; 40.7] in donors and 7.1 $\mu\text{g/L}$ [-2.4; 16.7] in non-donors).

Linear mixed models revealed no significant longitudinal associations between any of the iron parameters and cognitive functioning (Table 4), implying that the rate of cognitive change over 3 years did not vary according to body iron concentrations. However, stratifying our analyses by donor status indicated that higher serum iron significantly predicted less decline in sensorimotor speed over 3 years in non-donors (parameter estimate = 0.005, $p=0.010$, as compared with parameter estimate = -0.001, $p=0.253$ in blood donors), and less decline in word fluency over 3 years in blood donors (parameter estimate = 0.006, $p=0.012$, as compared with parameter estimate = -0.004, $p=0.165$ in non-donors), indicating effect modification by donor status. Stratification by sex or *APOE* E4 carrier status did not reveal any differences between men and women, or between carriers and non-carriers of the *APOE* E4 allele. Overall, the longitudinal associations between iron parameters and cognitive performance in the analyses stratified by sex, donor status, or *APOE* E4 carrier status were fairly inconsistent in terms of size and direction.

Associations between the *HFE* C282Y mutation and cognitive functioning

To investigate the influence of lifelong exposure to elevated iron levels on cognitive performance in later life, we assessed the associations between the *HFE* C282Y mutation and cognitive functioning on each of the five domains. The *HFE* C282Y mutation was not associated with cognitive performance in both the cross-sectional and longitudinal analyses (Table 5).

DISCUSSION

In the present study, none of the serum iron parameters, nor *HFE* C282Y genotype, were related to cognitive performance over 3 years of follow-up. However, the results from the cross-sectional analyses suggest that in older individuals serum ferritin and serum iron may be negatively related to the speed of cognitive functioning. Whereas higher ferritin concentrations were associated with decreased cognitive functioning across the three different speed measures at baseline, serum iron appeared to be negatively related to sensorimotor speed only. Memory processes, on the other hand, did not seem to be related to ferritin, nor any other serum iron parameter. In addition, non-transferrin bound iron and the *HFE* C282Y mutation were not associated with cognitive functioning in the cross-sectional analyses.

Carriers of the *HFE* C282Y mutation tend to show higher body iron concentrations than non-carriers (28). As genetic factors cannot be influenced by cognitive functioning, investigating the associations between *HFE* genotype and cognitive performance has the benefit of reducing confounding and ruling out the possibility of reverse causation (57). In the present study, we found that the *HFE* C282Y mutation was associated with significantly increased concentrations of serum iron and non-transferrin bound iron, as well as a statistically non-significant increase in serum ferritin. Contrary to expectation, we did not find any associations between *HFE* C282Y genotype and cognitive functioning. A possible explanation for the lack of such a relationship is the relatively small percentage of carriers of the *HFE* C282Y mutation (10.5%), which might have reduced the probability of detecting potential associations. In addition, no data were available concerning another common polymorphism of the *HFE* gene, H63D, which has been found to interact with the *HFE* C282Y genotype in determining individual iron levels (28, 58). Future studies investigating the associations between *HFE* genotype and cognitive functioning might increase statistical power by including both polymorphisms of the *HFE* gene in their analyses. Furthermore, it

should be noted that the generalizability of our study was limited by the nature of the study population; the present study sample was not representative of the general population, as participants had slightly elevated plasma total homocysteine concentrations and were vitamin B12 replete.

Non-transferrin bound iron has been hypothesized to be involved in neurodegenerative disorders characterized by iron deposition in the brain (44, 59). The putative relationship between non-transferrin bound iron and cognitive performance, however, has not been investigated before. The present results do not offer support for an association between non-transferrin bound iron and cognitive performance or age-related cognitive decline. Serum non-transferrin bound iron concentrations are generally very low in healthy individuals, while individuals heterozygous for the *HFE* C282Y mutation show slightly elevated non-transferrin bound iron concentrations (60). In hemochromatosis homozygotes, non-transferrin bound iron is typically present in much larger amounts (60). However, as the present study included only two individuals homozygous for the *HFE* C282Y mutation, the range of serum non-transferrin bound iron concentrations might have been too small for any associations with cognitive performance to become manifest. In addition, non-transferrin bound iron was only measured at baseline, thereby limiting the interpretation of the longitudinal analyses investigating the association of this iron parameter with cognitive functioning over 3 years.

Although elevated serum ferritin concentrations generally reflect increased body iron stores (42), serum ferritin levels also tend to be higher in conditions of inflammation, as ferritin is an acute-phase reactant (23, 51). In the present study, we controlled for this confounding effect of inflammation by including serum C-reactive protein concentration as a covariate in the statistical models. Post-hoc analyses showed that the inverse relationship between ferritin and cognitive speed was independent of C-reactive protein concentration, indicating that the observed cross-sectional association between serum ferritin and cognitive

speed could not be attributed to the presence of inflammation.

The present finding that higher serum ferritin levels were related to slower sensorimotor speed in carriers, but not in non-carriers of the *APOE* E4 allele, suggests that *APOE* E4 carrier status may modify the effect of elevated body iron stores on cognitive functioning. Although *APOE* E4 carrier status has been implicated in age-related cognitive decline and neurodegenerative processes (61, 62), it is unclear which mechanisms might be responsible for the putative interplay between *APOE* E4 carrier status and serum ferritin in affecting cognitive performance.

To date, only a few other population-based studies have investigated the relationship between iron parameters and cognitive performance in older individuals. These cross-sectional studies have yielded conflicting results. Whereas Gao and coworkers did not find any associations between serum iron and cognitive performance in a relatively small sample of 94 men and 94 women (20), La Rue et al. reported a positive correlation between serum transferrin and cognitive functioning, i.e. memory, visuospatial skills, and abstract reasoning, in a small sample of 67 men and 70 women (21). However, this association was not corrected for potential confounders, such as age, sex, BMI, and alcohol consumption.

A recent cross-sectional study performed by Lam et al. in a large population-based sample consisting of 602 men and 849 women, has shown an inverse linear association between serum iron and performance on memory tests in older women (22). Interestingly, in older men, Lam et al. found an inverted U-shaped relationship between serum iron and memory performance. Since we hypothesized that both low and high body iron levels would be associated with cognitive impairment, we also expected to find an inverted U-shaped relationship between body iron levels and cognitive performance. However, although the number of men in our sample ($n=586$) was comparable to the number of men included in the study by Lam and colleagues, thereby yielding similar statistical power, no curvilinear

associations became apparent between the iron parameters and cognitive functioning in the present study, neither in the total sample, nor after stratification by sex. The lack of curvilinear associations in our study may be due to the relatively small percentage of individuals with body iron levels below or above the normal limits. Therefore, possible non-linear associations between iron parameters and cognitive functioning may have remained undetected. Indeed, serum iron concentrations in the study by Lam et al. showed higher means and a broader range as compared with our study ($21.7 \pm 7.2 \mu\text{mol/L}$ in men and $19.4 \pm 6.2 \mu\text{mol/L}$ in women, as compared with $19.0 \pm 6.8 \mu\text{mol/L}$ in men and $17.4 \pm 4.7 \mu\text{mol/L}$ in women in our study).

It is worth noting that the prevalence of depleted iron stores in our study sample was slightly higher than that reported in other population-based studies with a similar age range (i.e. 5.3% as compared to 0.3-3%) (6, 27). In addition, our study showed a lower percentage of ferritin levels above the upper normal limit than other studies performed in older community-dwelling individuals, which used similar or even higher serum ferritin cutoff values (i.e. 6.5% as compared with 12-20%) (6, 27). This may be explained by the large number of blood donors in our study sample, as regular whole blood donation has been shown to lower body iron concentrations (8). Indeed, whereas 0.8% of non-donors showed ferritin levels below the lower normal limit and 13.1% showed ferritin levels above the upper normal limit, the prevalence among blood donors was 11.3% and 0.9%, respectively.

Upon stratification of the cross-sectional analyses by donor status, we found that higher serum iron was associated with decreased sensorimotor speed in non-donors, but not in blood donors. When the longitudinal analyses were stratified by donor status, we found that higher serum iron not only predicted less decline in sensorimotor speed over 3 years in non-donors, but also less decline in word fluency over 3 years in blood donors. Although these findings are not unequivocal, they do suggest that donor status may influence the nature of the

associations between serum iron parameters and cognitive performance. The underlying mechanism has yet to be elucidated.

We observed a significant increase in serum ferritin concentrations over the 3-year follow-up period, which is consistent with earlier reports indicating that body iron stores increase with aging (26). Although the difference was not statistically significant, serum ferritin showed a greater 3-year increase in donors as compared with non-donors. This difference may be related to the potential discontinuation of blood donation in regular whole blood donors during the course of the study. Unfortunately, we were not able to verify this assumption, as we did not monitor donor status during the 3-year follow-up period. Serum iron tended to decrease during the follow-up period, but this change was not statistically significant. Although the factors underlying the 3-year change in serum iron are not exactly clear, it is worth noting that they are not likely to be due to diurnal variation in serum iron levels (63), as we collected the 3-year blood samples at the same time of day as the baseline samples.

In the present study, we used serum iron parameters as a proxy measure for brain iron levels, even though the exact correlation between central and peripheral iron levels is unclear. Nonetheless, epidemiological studies have found evidence for a positive association between the two, by showing a significant correlation between iron concentrations in serum and cerebrospinal fluid in older individuals (64). On the other hand, it has also been suggested that brain iron levels may be relatively well-isolated from peripheral iron levels, for example in carriers of the *HFE* C282Y mutation (65). Thus, given the inability to measure cerebrospinal fluid or brain iron levels in volunteers, the use of serum iron parameters should be considered a shortcoming.

The main strengths of the present study are the use of longitudinal data, the measurement of several iron parameters as well as *HFE* C282Y genotype, the use of a large

community-based sample, and a very low attrition rate in the longitudinal phase (2%) (34). In addition, the cognitive test battery administered in the present study has been proven a sensitive and robust tool for detecting subtle changes in specific domains of cognitive functioning (35-39). Furthermore, the inclusion of both men and women, as well as blood donors and non-donors in our study, enabled us to investigate the relationship between iron parameters and cognitive functioning within these individual groups. The relevance of taking into account donor status in population-based studies is emphasized by the present finding that regular whole blood donation may modify the associations between iron parameters and cognitive performance. Indeed, the lack of documenting donor status might have confounded several other population-based studies investigating these associations, including those performed by Lam et al. (22), Gao et al. (20), and La Rue et al. (21).

In conclusion, the lack of any longitudinal associations between iron parameters and cognitive performance, as well as the lack of a relationship between *HFE* C282Y genotype and cognitive functioning, suggests that there is no clear-cut relationship between serum iron parameters and cognitive functioning or age-related cognitive decline in older community-dwelling individuals. However, the present study offers indications for a more intricate relationship between body iron levels and cognitive performance, which may be present only in a subsample of the community, e.g. in individuals carrying the *APOE* E4 allele.

To our knowledge, our study is the first to address the putative associations between the *HFE* C282Y mutation and cognitive performance in healthy older individuals, as well as the possible interactions between iron parameters and *APOE* E4 carrier status or donor status in relation to cognitive functioning. Prospective studies using large population-based samples are needed to further investigate the associations between iron parameters and cognitive performance, and to establish whether this association may differ across selected groups, e.g. based on sex, donor status, *APOE* E4 carrier status, or other risk factors associated with

cognitive decline. In addition, more research is necessary to identify the exact mechanisms by which iron parameters may influence cognitive functioning.

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