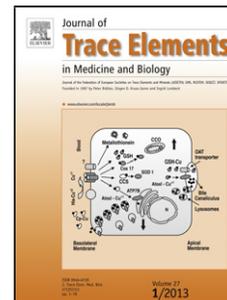


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Association of iron levels in hair with brain structures and functions in young adults

Short title: Iron, brain structures, and perfusion

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Abstract

BACKGROUND: Iron plays a critical role in normal brain functions and development, but it has also been known to have adverse neurological effects.

METHODS: Here, we investigated the associations of iron levels in hair with regional

gray matter volume (rGMV), regional cerebral blood flow (rCBF), fractional anisotropy (FA), mean diffusivity (MD), and cognitive differences in a study cohort of 590 healthy young adults.

RESULTS: Our findings showed that high iron levels were associated with lower rGMV in areas including the hippocampus, lower rCBF in the anterior and posterior parts of the brain, greater FA in areas including the part of the splenium of the corpus callosum, lower MD in the overlapping area including the splenium of the corpus callosum, as well as greater MD in the left hippocampus and areas including the frontal lobe.

CONCLUSION: These results are compatible with the notion that iron plays diverse roles in neural mechanisms in healthy young adults.

Keywords: Hair minerals, iron, regional gray matter volume, hippocampus, diffusion tensor imaging, perfusion

Introduction

Iron is an element that is essential to the body and the brain. Iron deficiency leads to poorer cognitive performance [1] In addition, our previous study showed that hair iron levels were associated with daily physical activity levels, novelty seeking, and extraversion; novelty seeking and extraversion have been associated with dopaminergic function [2].

Iron plays important and independent roles in a wide range of neurological and physiological processes. First, iron plays an essential role in the dopaminergic system because iron is required by tyrosine hydroxylase for dopamine synthesis [3](a).

Second, iron is plays important roles in the progression of neural aging and neurological

disorders such as Alzheimer's disease because iron accumulates in some brain regions, such as the hippocampus, generating a surplus of reactive ions that damage neural tissues [4] (b). In the elderly, a greater iron concentration in the hippocampus is consistently associated with lower hippocampal volume [5]. Third, iron is critical for the formation of hemoglobin and red blood cells, which transport oxygen to the body [6] and the brain [7], thereby affecting cerebral blood flow (CBF) [8](c). Finally, iron plays a critical role in myelin formation during development [7](d).

In the present study, we aimed to reveal how the iron level in the body associates with neural measures—possibly through diverse independent mechanisms. For the process (a), we focused on the mean diffusivity of diffusion tensor imaging (DTI) in the dopaminergic system (MDDS), which reflects properties of the dopaminergic system [9]. For the process (b), we focused on the regional gray matter volume (rGMV) with a particular focus on the hippocampus. For the process (c), we focused on rCBF. For the process (d), we focused on the fractional anisotropy (FA) of DTI, which is affected by myelination processes [10].

Hair mineral analysis is particularly useful for investigating body mineral levels because these levels are not affected by rapid fluctuations in mineral intake and show long-term stability [11]. Concentrations of basic elements in the body and hair are substantially correlated [12], although it was suggested that to get comparable results in hair mineral analysis, sampling by trained personnel, standardized pre-analytical and analytical procedures, using suitable and sensitive equipment were required [13].

Methods

Subjects

Data from 590 healthy right-handed individuals (438 men and 152 women; mean age = 20.87 ± 1.94 years, range 18-27 years) were used in this study as part of an ongoing project investigating associations among brain imaging, cognitive functions, aging, genetics, and daily habits. Hair samples were collected from the large study samples, but data from subjects who had undergone hair dying, bleaching, or a permanent wave within the last 6 months were excluded from the present study to improve data quality, as the iron content may have been affected by the color due to hair dying. This procedure of exclusion is in accordance with our previous study [2]. As a result, only data from abovementioned 590 subjects were analyzed in this study. All subjects were university, college, or postgraduate students or subjects who had graduated from these institutions within 1 year before the experiment. All subjects had normal vision. None had a history of neurological or psychiatric illness. A history of psychiatric illness and/or recent drug use was assessed using our laboratory's routine questionnaire in which each subject answered questions relating to their current or previous experiences of any of a list of illnesses and drugs taken recently. Handedness was evaluated using the Edinburgh Handedness Inventory [14]. Written informed consent was obtained from each subject in accordance with the Declaration of Helsinki (1991). For subjects whose age is < 20 , written informed consent was obtained from their parents or guardians. This study was approved by the Ethics Committee of Tohoku University.

Subjects were instructed to get sufficient sleep, maintain their condition, eat sufficient breakfast, and consume their normal amount of caffeinated foods and drinks on the day of cognitive tests and MRI scans. In addition, subjects were instructed to avoid alcohol the night before the assessment. For limitations of this study related to

subject characteristics, see **Supplemental Discussion**. The descriptions in this subsection were mostly reproduced from our previous study [2, 15].

Hair acquisition and hair mineral analysis

Scalp hair samples (approximately 4 cm in length, 0.1 g in weight) were collected from each subject, with the hair cut as close to the scalp as possible. The hair samples were sent to a research laboratory (La Belle Vie Inc., Tokyo, Japan) and analyzed by established methods [16-21], as described below.

Hair samples of 75 mg were weighed into 50-ml plastic tubes and washed twice with acetone and then with 0.01% Triton solution, in accordance with the procedures recommended by the Hair Analysis Standardization Board [22]. The washed hair sample was mixed with 10 ml of 6.25% tetramethylammonium hydroxide (TMAH, Tama Chemical, Tokyo, Japan) and 50 μ l of 0.1% gold solution (SPEX Certi Prep, Metuchen, NJ, USA) and then dissolved at 75°C with shaking for 2 h. After cooling the solution to room temperature, an internal standard (Sc, Ga, and In) solution was added, and, adjusting its volume gravimetrically, the obtained solution was used for mineral analysis. The mineral concentrations were measured with inductively coupled plasma mass spectrometry (Agilent-7500ce; Agilent Technologies, Tokyo, Japan) using the internal standard method [16, 23, 24] and are expressed as μ g/g hair (ppm). For quality control, human hair certified reference materials supplied from the National Institute for Environmental Studies of Japan (Tsukuba, Japan; NIES CRM no. 13) [25] were used.

For statistical analysis, iron was converted to logarithms for use in the analyses, as reported in previous studies [2, 16, 19]. The raw data as well as the data of logarithms did not conform to normality (Shapiro-Walk test, $p < 0.05$).

The hair iron level is reduced in children with iron-deficiency anemia [26] and the greater hair iron level is associated with greater physical activity level [2] and has validity.

The descriptions in this subsection were reproduced from our previous study [2], which used the exact methods.

Psychological measures

The following psychological measures were administered. The descriptions in this subsection have been largely reproduced from our previous work [2].

[A] The SA creativity test [27], which measures creativity through divergent thinking, involves three types of tasks: generate unique ways of using typical objects, imagine desirable functions for ordinary objects, and imagine the consequences of unimaginable things happening [28].

[B] The Novelty Seeking Scale, Japanese version [29], of the Temperament Character Inventory [30] was used to measure novelty seeking tendency. [C] The vigor subscale of the shortened Japanese version [31] of the Profile of Mood States (POMS) [32], which measures participants' motivation was used to measure each participant's mood on the day of the experiment [33]. [D] The Extraversion Scale on the Japanese version of the NEO Five-Factor Inventory (NEO-FFI) [34] was used to measure extraversion. [E] Data on the subjects' average daily physical activity levels during the past 1 month were collected using a self-report questionnaire. The questionnaire consisted of multiple choice questions with the following four response options: 1) little; 2) have exercised a little (approximately 1000 steps or 400 m); 3) have exercised modestly (approximately 1000–4000 steps or 400–1600 m); or 4) have exercised very much (more than 4000 or

1600 m).

Image acquisition

The methods for magnetic resonance imaging (MRI) acquisition were described in our previous study and are reproduced below [35, 36]. All MRI data acquisition was performed using a 3T Philips Achieva scanner (Philips, Best, The Netherlands). High-resolution T1-weighted structural images (240×240 matrix, TR = 6.5 ms, TE = 3 ms, FOV = 24 cm, slices = 162, slice thickness = 1.0 mm) were collected using a magnetization-prepared rapid gradient echo sequence. VBM analyses were performed in 590 subjects.

ASL was performed to measure resting state CBF. As described elsewhere [33], a pulse ASL method was used with quantitative signal-intensity targeting by alternating the radio-frequency pulse labeling of arterial regions (QUASAR) [37]. Details of the sequence and method for calculating perfusion parameters have been outlined elsewhere [37-39]. The actual imaging parameters were as follows: 64×64 matrix, TR = 300 ms, TE = 22 ms, FOV = 24 cm, seven slices, slice thickness = 7 mm (2.0 mm gap), SENSE = 2.5, 84 averages, and scan duration = 5 min 52 s. We determined the position of the slice by putting the fourth of seven slices on the body of the corpus callosum in the coronal scout view [40]. During ASL scanning, subjects were instructed to remain as motionless as possible with their eyes closed and not to sleep or think about anything in particular. Maps of raw resting rCBF and the longitudinal relaxivity (R1) of each subject were obtained using dedicated software running on IDL (Research Systems, Boulder, Colorado) [37; National Neuroscience Institute, Singapore]. The following constants were used in the CBF calculation: T1 of arterial blood, 1.65 s; inversion

efficiency, 95%; and blood–brain partition coefficients for GM and WM of 0.98 and 0.82, respectively [37].

Diffusion-weighted data were acquired using a spin-echo EPI sequence (TR = 10293 ms, TE = 55 ms, FOV = 22.4 cm, $2 \times 2 \times 2$ mm³ voxels, 60 slices, SENSE reduction factor = 2, number of acquisitions = 1). The diffusion weighting was isotropically distributed along 32 directions (b value = 1,000 s/mm²). Additionally, three images with no diffusion weighting (b value = 0 s/mm²; b = 0 images) were acquired using a spin-echo EPI sequence (TR = 10293 ms, TE = 55 ms, FOV = 22.4 cm, $2 \times 2 \times 2$ mm³ voxels, 60 slices). FA and MD maps were calculated from the collected images using a commercially available diffusion tensor analysis package on the MR console. For more details, see **Supplemental Methods**. The rCBF analyses were performed for 518 subjects whose rCBF maps were successfully obtained; other analyses were performed for all 590 subjects.

Preprocessing of T1-weighted structural data.

Preprocessing of the structural data was performed using Statistical Parametric Mapping software (SPM12; Wellcome Department of Cognitive Neurology, London, UK) implemented in MATLAB (Mathworks Inc., Natick, MA, USA). Using the new segmentation algorithm and diffeomorphic anatomical registration through the exponentiated lie algebra (DARTEL) registration process implemented in SPM12, the T1-weighted structural images of each individual were segmented and normalized to the Montreal Neurological Institute (MNI) space to generate images with $1.5 \times 1.5 \times 1.5$ mm³ voxels. In addition, we performed a volume change correction (modulation) [41]. Subsequently, generated rGMV images were smoothed by convolving them with an

isotropic Gaussian kernel of 8-mm full-width at half-maximum (FWHM). For a full description of these procedures, see the **Supplemental Methods**. The description in this paragraph was mostly reproduced from our previous study that used the same method [42].

Preprocessing of perfusion data.

Preprocessing of rCBF images was performed with the same procedure as that in our previous study [36]. Briefly, using the R1 map, we realigned rCBF images to the T1-weighted structural image of each individual. Then, using the normalization parameter of the T1-weighted structural image derived from the VBM process, rCBF images were normalized to the MNI space to generate images with $1.5 \times 1.5 \times 1.5 \text{ mm}^3$ voxels. The normalized image was masked by the mask image of areas where the images of all subjects of the template have signals. Lastly, the resultant images were smoothed by convolving them with an isotropic Gaussian kernel of 8-mm FWHM. For details, see **Supplemental Methods**.

Preprocessing of diffusion data.

MD, measured using DTI, is a direction-independent measure of how water molecules freely move. It is considered to reflect the amount of tissue in the brain, which prevents the free movement of water; differences in MD may thus reflect tissue changes, such as astrocyte swelling, synaptic changes, dendritic spine changes, and angiogenesis. Differences in MD are also sensitive to neural plasticity [43-45]. MD in the dopaminergic system areas (MDDS) is sensitive to the pathology of dopaminergic systems compared with other imaging modalities (Parkinson's disease) [46, 47]. MDDS

is sensitive to treatment with the dopamine agonists used to manage this pathology [48] as well as to the cognitive states and traits associated with the function of the dopaminergic system [49, 50].

Preprocessing and analysis of diffusion data were performed using SPM8 implemented in MATLAB. Briefly, we normalized FA and MD images of subjects with previously validated DARTEL-based registration process method to obtain images with $1.5 \times 1.5 \times 1.5 \text{ mm}^3$ voxels. Next, for MD images, tissues unlikely to be gray or white matter were carefully removed and smoothed by convolving them with an isotropic Gaussian kernel of 8-mm FWHM. For FA images, tissues unlikely to be white matter were carefully removed and smoothed by convolving them with an isotropic Gaussian kernel of 6-mm FWHM. For details, see **Supplemental Methods**. The description in this subsection was mostly reproduced from our previous study that used the same method [49]

Statistical analysis

The relationships among psychological variables and mineral levels were investigated using multiple regression analysis in SPSS 22.0 (SPSS Inc., Chicago, IL). We investigated the associations of hair iron levels with SA creativity test scores, POMS Vigor Subscale scores, Novelty Seeking Scale scores, Extraversion Scale scores, and physical activity levels. Each multiple regression analysis included age, sex, self-reported height, and body mass index (BMI), which were calculated from the self-reported height and weight and hair iron levels as independent variables and the dependent variable was one of five psychological variables (SA creativity test scores, POMS Vigor Subscale scores, Novelty Seeking Scale scores, Extraversion Scale scores,

and physical activity levels). As a result, we conducted five separate multiple regression analyses. These are replication analyses from our previous study, but with an increased sample size of the analysis and the descriptions of this subsection were largely reproduced from our previous study [2].

Statistical analyses of imaging data were performed with SPM as whole-brain multiple regression analyses. We investigated if rGMV, rWMV, rCBF, FA, and MD were associated with individual differences in hair iron levels. These analyses included sex, age, self-reported height, and BMI, which were calculated from self-reported height and weight and iron levels in hair. In the analysis of rGMV and rWMV, we included only voxels with an rGMV or rWMV signal intensity of >0.05 for all participants. This threshold value (0.05) is a widely used value that has been reported in numerous previous VBM studies [51-58]. The analyses of rCBF, FA, and MD were limited to the masks of each modality created as described above. A multiple comparison correction was performed using the t-score with randomized (5,000 permutations) nonparametric testing using the toolbox (<http://dbm.neuro.uni-jena.de/tfce/>). We applied a voxel threshold using a family-wise error corrected at $P < 0.05$ (an extent threshold is not considered in this statistical test). Since this test is a non-parametric test, it is generally robust to effects of outliers. Although, the raw data as well as the data of logarithms did not conform to normality as described above, the permutation-based statistics in the main imaging analyses are valid under the data of the non-normality [59]. This correction accounts for multiple comparisons without the need to apply an arbitrary threshold of the extent threshold. Because this controls for multiple comparisons using the height threshold based on permutation tests, the voxel level threshold is much more severe than the widely used cluster size tests [60]. Thus, using a more lenient threshold

such as $p < 0.001$, uncorrected, the significant areas would be larger.

Results

Basic data

Table 1 shows the average \pm standard deviation (SD) for age, psychological variable scores, and iron levels for all study participants. Figure 1 shows the distribution of the iron levels. The average, SD, minimum, maximum, median, 25 percentile, and 75 percentile of hair iron level (ppm) was 5040.4, 1235.4, 2668, 16580, 4856.7, 4261.6, and 5472.6, respectively. There were no significant sex differences of raw hair iron level (t-test, $p > 0.05$).

Associations between psychological variables and hair iron levels.

Multiple regression analyses correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with the greater POMS Vigor Subscale score, greater daily physical activity level, and greater extraversion. They further revealed near significant tendencies ($0.05 < P < 0.07$) of associations between greater hair iron levels and greater creativity (measured by a divergent thinking test) as well as greater Novelty Seeking Scale scores.

For the statistical values of all results, please refer to Table 2.

Regional gray matter volume and regional white matter volume analyses

Whole-brain multiple regression analyses correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with less rGMV in the area of the right thalamus and right hippocampus (Fig. 2). There were

no significant associations between hair iron levels and rWMV. For the statistical significance values of all results, please refer to Table 3.

Regional cerebral blood flow analysis

Whole-brain multiple regression analysis correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with less rCBF during rest in the area of the left medial frontal gyrus and the area of the right middle cingulate gyrus (Fig. 3). For the statistical significance values of all results, please refer to Table 3.

Regional fractional anisotropy and mean diffusivity analyses

Whole-brain multiple regression analyses correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with greater FA in the area adjacent to the right thalamus as well as in the area of the splenium of the corpus callosum (Fig. 4). For the statistical significance values of all results, please refer to Table 3.

Whole-brain multiple regression analyses correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with greater MD in areas in the left hippocampus, left parahippocampal gyrus, brainstem, left precentral gyrus, left postcentral gyrus, and left lateral prefrontal cortex (Fig. 5). Furthermore, whole-brain multiple regression analyses correcting for the effects of age, sex, height, and BMI revealed that greater hair iron levels were significantly associated with less MD in the right thalamic radiation, areas of the splenium of the corpus callosum, right occipital lobe, right fusiform gyrus, right superior, and right middle

temporal gyrus (Fig. 6). The significant associations between hair iron levels and MD in splenium of the corpus callosum included the areas of significant associations between hair levels and FA. For the statistical significance values of all results, please refer to Table 4.

Discussion

To the best of our knowledge, this is the first study to investigate the associations between hair iron levels and brain imaging measures in young adults. Greater hair iron level was associated with less rGMV, less rCBF, and greater FA as well as greater MD and less MD in different areas (but not associated with MD in the areas associated with the dopaminergic system). We assumed different and independent neural mechanisms behind these relationships, reflecting the diverse physiological roles of iron.

Regional volume analyses

Hair iron levels were negatively associated with rGMV in areas including parts of the right hippocampus and right thalamus. In addition, the associations between greater hair iron levels and greater MD were seen in the left hippocampus and left hippocampal gyrus. Associations between iron and the hippocampus have been indicated in previous studies as follows. In Alzheimer's disease, the ferritin iron level in the hippocampus was reported to be elevated [61]. Also, CSF ferritin levels predict greater age-related decline in hippocampal volume in the elderly and predict Alzheimer's disease outcomes [62]. The present study supports these previous findings and demonstrated that even in young adults, body iron levels are associated with hippocampal volume. The mechanism of association is unclear, although the

hippocampus is one area where iron tends to accumulate [63]. It has been suggested that the aggregation of the tau protein, which is critical for the removal of iron and is important in the development of Alzheimer's disease, may be related to associations between hippocampal atrophy and iron levels [64]. Alternatively, neuro-oxidative stress caused by free iron may contribute to atrophy and lead to lower rGMV in the hippocampus. These mechanisms may also contribute to greater MD in the left hippocampus in subjects with higher hair iron levels, as greater MD is thought to reflect less tissue and more water [9].

rCBF analyses

Hair iron levels were negatively associated with rCBF during rest in areas of the anterior and posterior parts of the brain. As described in the Introduction, iron is critical to the formation of hemoglobin, which is critical for the transportation of oxygen to the body and brain [7]. When oxygen is fully supplied and binds to heme iron in hemoglobin, this leads to the contraction of blood vessels [65]. Therefore, more iron from diet may lead to less CBF. In animal experiments with artificially reduced arterial hemoglobin content, CBF was reported to increase in proportion to the reduction in hemoglobin [8]. However, as are the cases of analyses of other types of brain images, the reason why only parts of the brain showed significant associations remains unclear. One possible reason is that areas of significant associations might be the so-called "tip of the iceberg" caused by statistical fluctuation and may not have a significant meaning. Alternatively, it is known that some brain regions, including areas of the medial parietal lobes, show high rCBF at rest [66], and such regions may be sensitive to changes of rCBF in statistical analyses. Finally, cognitive differences associated with hair iron

level, as identified in the psychological analyses, may be directly associated with rCBF at rest. However, greater novelty seeking and creativity—which showed tendencies of associations with greater hair iron level—showed significant associations with greater rCBF in the medial PFC and medial parietal cortex, respectively [33, 67], and this interpretation is not consistent with previous studies. Future studies may need to confirm these associations.

FA analyses

Hair iron levels were positively associated with FA measures of DTI. FA is a measure of the degree of anisotropy and is thought to be modulated by the degree of myelination, axonal membrane thickness and diameter, and/or the amount of parallel organization of axons [10, 68]. Iron deficiency, even after the postweaning period, is associated with hypomyelination [69]. The amount of myelin is closely associated with the expression of transferrin (the main protein in blood that binds to iron and transports it throughout the body) by oligodendrocytes (highly specialized neural cells whose function is to myelinate the central nervous system axons) [70]. The production of transferrin is under transcriptional control and is responsive to the iron status of the cell [70]. When iron levels become too high, oligodendrocytes are particularly vulnerable to death by oxidative damage. Therefore, proper iron levels are important for appropriate brain states [71]. The sources of regional specificity of the areas of significant associations are not clear and may be the “tip of the iceberg” caused by statistical fluctuation and not be meaningful. However, in areas where fibers cross, the associations between myelination and FA become unclear [72], and the corpus callosum (which does not have crossed fibers) may be sensitive to the findings.

MD analyses

Greater hair iron level was associated with greater MD in areas of the left hippocampus and frontal areas, whereas it was associated with less MD in areas of the posterior parts of the brain. These findings are not consistent with our prediction, which was that greater hair iron level would be associated with less MD of the dopaminergic system as it was associated with greater novelty seeking [2], which has been shown to be associated with less MD [73]. Further, iron reduction leads to a reduction in dopamine D2 receptors and such changes in dopamine D2 receptors are thought to be associated with the reduction in motor and cognitive functions caused by iron restriction [74], while less MDDs associated with greater dopamine synthesis capacity [75]. The reasons for these inconsistencies are not clear. However, we suggest a few possibilities. First, the reasons for the associations between greater hair iron level and less MD may be the same as those for the associations between greater hair iron level and greater FA, i.e., they may be due to increased myelination facilitated by iron, as described above. Myelination is one factor that contributes to reduced MD values [44]. Consistent with this notion, the areas of associations between greater hair iron level and greater FA overlapped with areas of associations between greater hair iron level and less MD. On the other hand, in areas such as the hippocampus, which is an area where iron tends to accumulate [63], the neurological effects of higher hair iron level as described above may be more prevalent, which may be why areas in the left hippocampus showed associations between greater MD and greater hair iron level. The lack of significant associations in areas of the dopaminergic system may be because (a) the neurological effects that iron causes through neuro-oxidative stress [7] damage tissue and increase

MD and (b) the effects of iron to facilitate dopaminergic function [9] cancelled each other out. However, as the present study is not an interventional or longitudinal observational study, it is difficult to make inferences on the causal link. As a third possibility, genetic or environmental factors may affect all of the personality, brain mechanisms, and hair iron levels (rather than the iron intake leading to the observed psychological and neural properties). Future longitudinal studies are needed to address these issues. Further, despite previous evidence suggesting associations between MD of the dopaminergic system and alterations in the properties of the dopaminergic system [9], as well as the observed associations between hair iron level and extraversion and state associated with motivation (Vigor subscore of POMS), both of which are associated with dopaminergic functions in the present study [2], we did not find any significant associations between MDDS and hair iron level. Future studies using other modalities, such as positron emission tomography studies of dopamine receptors [76], could reveal the underlying neural mechanisms of these associations.

Psychological analyses

In our previous study [2] with a smaller but overlapping sample, greater hair iron levels were significantly associated with greater physical daily activity level (beta = 0.219, $p = 6.68 \times 10^{-5}$), extraversion (beta = 0.126, $p = 0.020$), and novelty seeking (beta = 0.112, $p = 0.039$), but the associations with creativity (beta = 0.047, $p = 0.392$) and the POMS Vigor Subscale score (beta = 0.072, $p = 0.194$) did not reach significance [2]. In the present study, the associations of hair iron levels with physical daily activity level (beta = 0.121, $p = 0.003$), extraversion (beta = 0.082, $p = 0.045$), and the POMS vigor subscale score (beta = 0.088, $p = 0.033$) were significant. But the associations of hair

iron levels with creativity (beta = 0.078, $p = 0.060$) and the novelty seeking (beta = 0.076, $p = 0.064$) were marginally insignificant ($0.05 < P < 0.07$). The results were mostly similar and not substantially different between the present study and the previous study. The statistical phenomenon called “regression to the mean” (the best performing samples and worst performing individual samples in the first measurement tend to be closer to the average in the second measurement) can explain some of the changes in the significance levels and effect sizes.

Limitations

There are a few limitations to this study that is common to those of our previous study [2]. As is the case for all cross-sectional studies of hair mineral analysis, we cannot prove causal effects. Thus, future longitudinal or interventional studies are needed. Further, previous studies suggested that the accumulation of iron in the brain is important for the progression of neurological diseases [4]. The associations of iron with brain imaging measures in such groups may be different from the present findings, and future studies are needed to investigate such issues. Finally, in the present study, as was the case in our previous study [2], subjects who had dyed their hair within 6 months of the study were excluded from the analysis. As a result, the sample was biased toward males and due to the relatively small number of females, we could not conduct sex-specific analyses. Future studies may need to investigate such issues.

Summary

In summary, we investigated the associations of hair iron levels with cognitive domains, brain structures and perfusion in a relatively large cohort of young adult subjects. As described in the Introduction, non-human studies have demonstrated the

important role of iron in a number of neural and physiological mechanisms, such as normal dopaminergic functions, hemoglobin formation, myelination, as well as neuro-oxidative stress and other neurological effects. Human studies have shown that higher body iron levels are associated with greater levels of states and traits associated with positive affect, attention to people, and dopaminergic function. However, in the elderly, a higher ferritin level was longitudinally associated with the risk of Alzheimer's disease and decline of hippocampal volume. The present study extended these findings and showed that even in young adults, a higher iron level was associated with reduced volume and greater diffusivity of an area including the hippocampus. Further, a higher iron level was associated with greater FA and lower rCBF in some regions, which is in line with physiological studies. Finally, higher iron levels were associated with lower MD in posterior areas including the splenium of the corpus callosum. These findings are congruent with the idea that iron has diverse effects in the brains of normal young adults.

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Journal Pre-proof

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Figure legends

Fig. 1. Histograms showing S-A creativity test scores and the hair iron levels for male and female subjects.

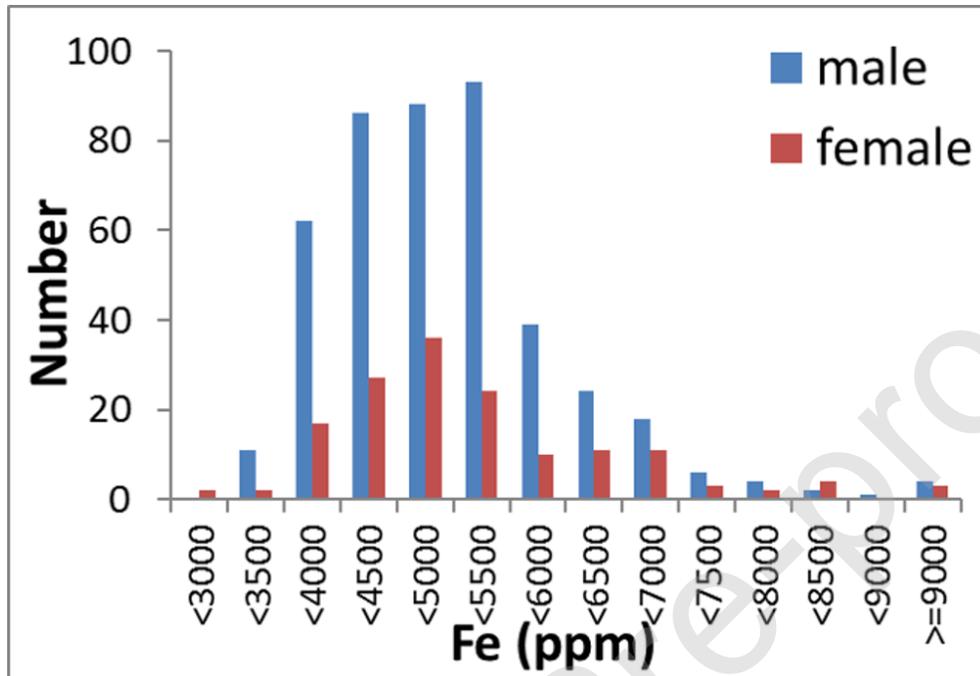


Figure 2. Associations between hair iron level and rGMV. Greater hair iron level was significantly associated with less rGMV in the area of the right thalamus and right hippocampus. Colored bars indicate T scores. All results are overlaid on a “single-subject T1” SPM8 image. (a): Areas of significant associations. In the left column, the

results shown were obtained using a threshold of $P < 0.05$, corrected for multiple comparisons based on 5000 permutations using the t-score. (b): In the right column, the results shown were obtained using a threshold of $P < 0.005$, uncorrected for visualization purposes. (c) A partial residual plot with the trend line depicting the associations between residuals in the multiple regression analyses with mean rGMV in the significant cluster as a dependent variable and the logarithms of hair iron levels and other confounding factors as independent variables; 95% confidence intervals for the trend line are shown.

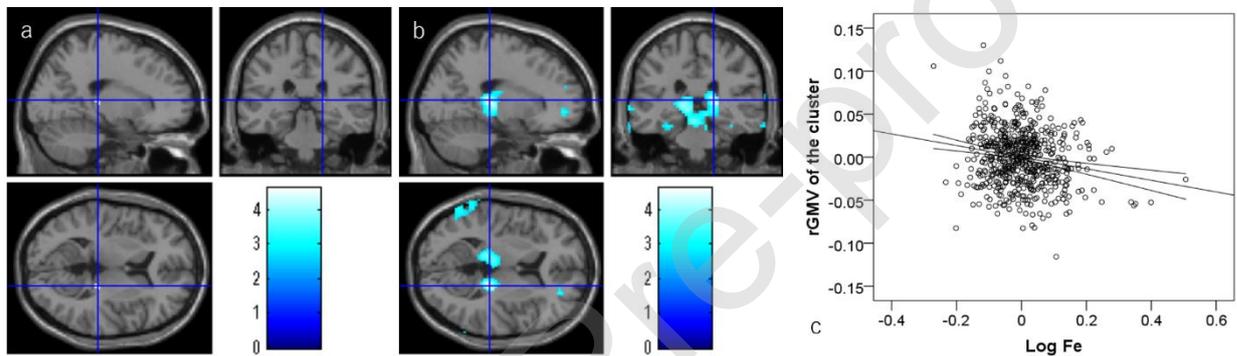


Figure 3. Associations between hair iron level and rCBF. Greater hair iron level was significantly associated with less rCBF in the areas of the left medial frontal gyrus and

right middle cingulate gyrus. Colored bars indicate T scores. All results are overlaid on a “single-subject T1” SPM8 image. (a): Areas of significant associations. In the left column, the results shown were obtained using a threshold of $P < 0.05$, corrected for multiple comparisons based on 5000 permutations using the t-score. (b): In the right column, the results shown were obtained using a threshold of $P < 0.005$, uncorrected for visualization purposes. (c, d) Partial residual plots with the trend lines depicting the associations between residuals in the multiple regression analyses with mean rCBF in the significant clusters of (c) the left medial frontal gyrus and (d) right middle cingulate gyrus as dependent variables and the logarithms of hair iron levels and other confounding factors as independent variables; 95% confidence intervals for the trend lines are shown.

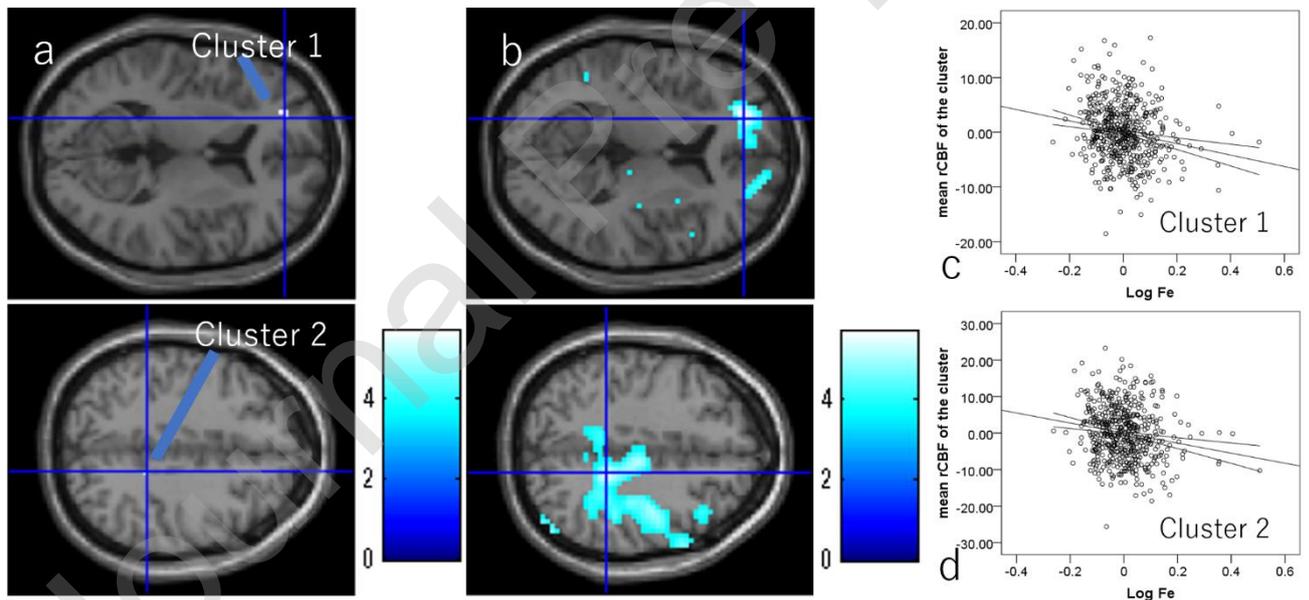


Figure 4. Associations between hair iron level and FA. Greater hair iron level was significantly associated with greater FA in the white matter area adjacent to the right thalamus and splenium of the corpus callosum. Colored bars indicate T scores. All results are overlaid on mean preprocessed, but not smoothed, FA images of a subset of participants of the project. (a): Areas of significant associations. In the left column, the results shown were obtained using a threshold of $P < 0.05$, corrected for multiple comparisons based on 5000 permutations using the t-score. (b): In the right column, the results shown were obtained using a threshold of $P < 0.005$, uncorrected for visualization purposes. (c, d) Partial residual plots with the trend lines depicting the associations between residuals in the multiple regression analyses with mean FA values in the significant clusters of (c) the splenium of the corpus callosum and (d) the white matter area adjacent to the right thalamus as dependent variables and the logarithms of hair iron levels and other confounding factors as independent variables; 95% confidence intervals for the trend lines are shown.

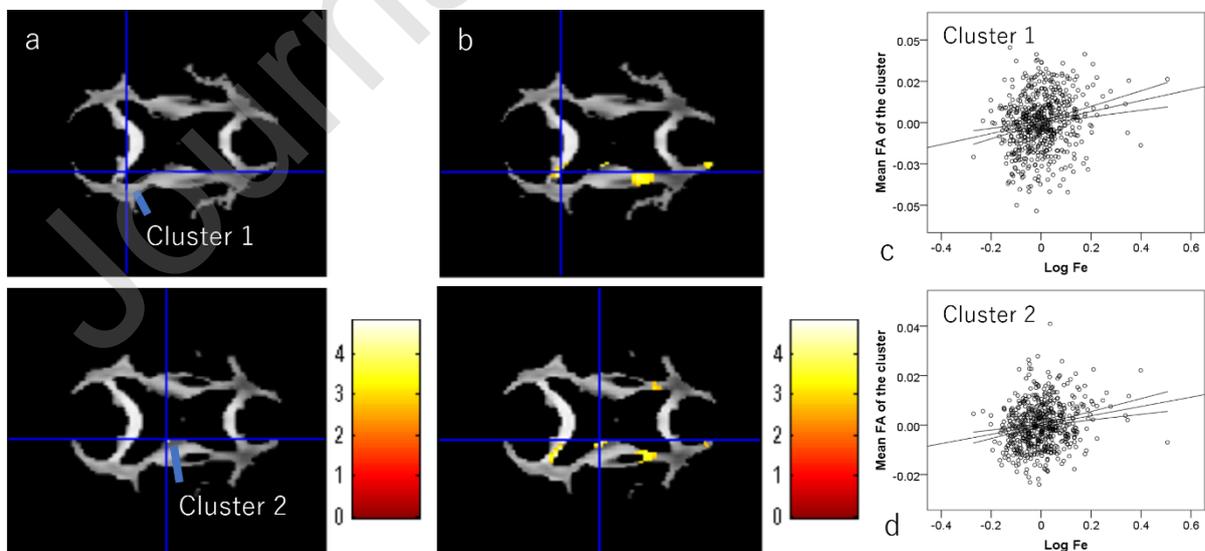


Figure 5. Associations between hair iron level and MD. Greater hair iron level was significantly and positively associated with greater MD in areas in the left hippocampus, brain stem, parahippocampal gyrus, right lateral prefrontal cortex, and right precentral and postcentral gyrus. Colored bars indicate T scores. All results are overlaid on a “single-subject T1” SPM8 image. (a): Areas of significant associations. In the left column, the results shown were obtained using a threshold of $P < 0.05$, corrected for multiple comparisons based on 5000 permutations using the t-score. (b): In the right column, the results shown were obtained using a threshold of $P < 0.005$, uncorrected. (c, d) Partial residual plots with the trend lines depicting the associations between residuals in the multiple regression analyses with mean MD values in the significant clusters of (c) the right precentral gyrus and (d) left hippocampus as dependent variables and the logarithms of hair iron levels and other confounding factors as independent variables; 95% confidence intervals for the trend lines are shown.

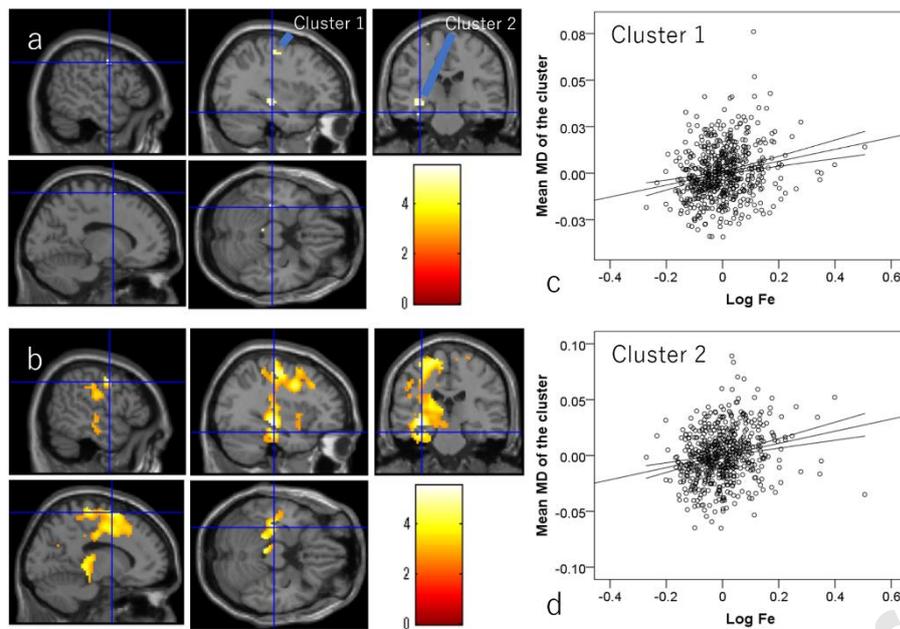


Figure 6. Associations between hair iron level and MD. Greater hair iron level was significantly associated with less MD in the areas of the right thalamic radiation, right occipital lobe, right fusiform gyrus, right superior, and middle temporal gyrus. Colored bars indicate T scores. All results are overlaid on a “single-subject T1” SPM8 image. (a): Areas of significant associations. In the left column, the results shown were obtained using a threshold of $P < 0.05$, corrected for multiple comparisons based on 5000 permutations using the t-score. (b): In the right column, the results shown were obtained using a threshold of $P < 0.005$, uncorrected for visualization purposes. (c) A partial residual plot with the trend line depicting the associations between residuals in

the multiple regression analyses with mean MD in the largest significant cluster as a dependent variable and the logarithms of hair iron levels and other confounding factors as independent variables; 95% confidence intervals for the trend line are shown.

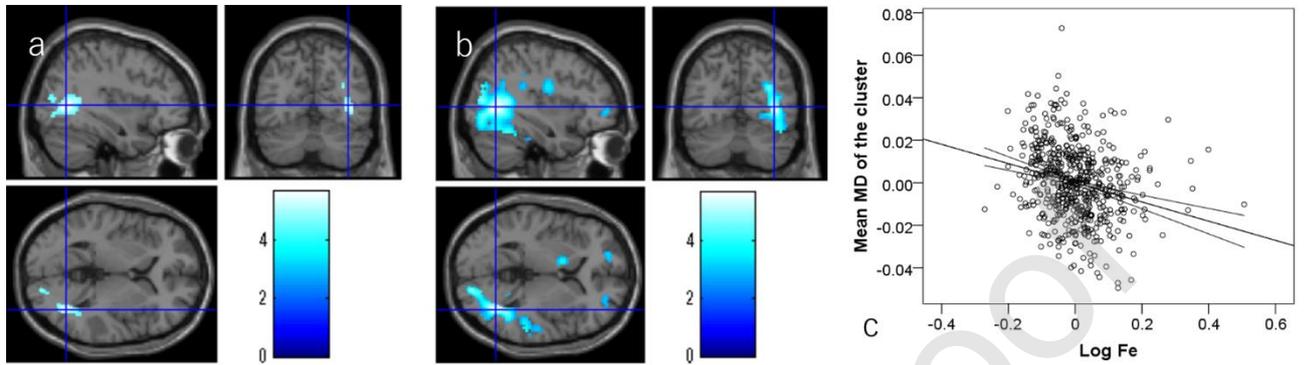


Table 1

Psychological variables and iron levels among the study participants

Measure	Mean	SD	range
Age (N = 590)	20.87	1.94	18-27
S-A creativity (N =590)	38.43	10.84	13-71
POMS Vigor (N = 581)	5.73	4.00	0-20
Novelty seeking (N = 590)	20.30	5.72	4-36
Extraversion (N = 590)	25.16	7.00	7-47
Physical activity level (N =590)	2.37	1.11	1-4
iron (ppm) (N = 590)	5040.4	1235.4	2668-16580

Table 2

Results of multiple regression analyses investigating associations of hair iron levels with psychological variables

Variables	The statistics of the predictors				The statistics of the models		
	Beta	Semi-partial correlation coefficients	P-value	T value	R	Adjusted R2	P-value
S-A creativity	0.078	0.077	0.060	1.884	0.152	0.015	0.018
POMS Vigor	0.088	0.088	0.033	2.141	0.199	0.031	<0.001
Novelty seeking	0.076	0.075	0.064	1.853	0.181	0.024	0.002
Extraversion	0.082	0.082	0.045	2.010	0.191	0.028	0.001
Physical activity level	0.121	0.120	0.003	2.935	0.177	0.023	0.002

Table 3. Brain regions with significant associations between hair iron level and rGMV, rCBF, and FA

Included gray/white matter areas	X	y	z	t-score	Corrected P-value (FWE)	Cluster size (voxels)
Associations between greater hair iron level and less rGMV						
Right thalamus/Right hippocampus	19.5	-30	7.5	4.63	0.019	35
Associations between greater hair iron level and less rCBF						
Left medial frontal gyrus	-21	48	9	4.19	0.023	26
Right middle cingulate gyrus	15	-39	42	3.96	0.048	1
Associations between greater hair iron level and greater FA						
N.A.	13.5	-16.5	12	4.83	0.008	5
Corpus callosum splenium	21	-45	15	4.74	0.024	3

Table 4. Brain regions with significant associations between hair iron level and MD

Included gray matter areas	x	y	z	t-score	Corrected P-value (FWE)	Cluster size (voxel)
Associations between greater hair iron level and greater MD						
Left precentral gyrus	-51	-1.5	42	5.55	0.003	44
Left hippocampus	-33	-26	-11	5.32	0.006	238
Brain stem	-4.5	-36	-19.5	5.12	0.010	15
Left postcentral gyrus	-21	-28. 5	64.5	5.01	0.013	47
Left precentral gyrus	-30	-15	52.5	4.84	0.021	92
Left parahippocampal gyrus	-36	-24	-22.5	4.81	0.023	18
Left superior frontal gyrus	-13. 5	3	67.5	4.67	0.036	11

Brainstem	-6	-31. 5	-9	4.58	0.048	1
Brain stem	-9	-30	-9	4.56	0.049	1
Middle frontal gyrus	-32	3	37.5	4.56	0.050	1
Associations between greater hair iron level and less MD						
Right posterior thalamic radiation	37.5	-64. 5	3	5.71	<0.001	1831
NA	31.5	-66	22.5	4.84	0.006	36
Right calcarine cortex	18	-90	3	4.76	0.008	139
Right fusiform gyrus	43.5	-75	-18	4.69	0.009	75
Right superior temporal gyrus	54	-40. 5	22.5	4.45	0.023	24
Right superior occipital gyrus	39	-79	23	4.26	0.044	1
Right fusiform gyrus	30	-82. 5	-19.5	4.25	0.046	1

Right cerebellum	40.5	-48	-27	4.23	0.048	1
Right middle temporal gyrus	55.5	-4.5	-33	4.21	0.050	1

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