

Iron levels in the human brain: a post-mortem study of age-related changes and anatomical region differences

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Abstract

The link between brain iron homeostasis and neurodegenerative disease has been the subject of extensive research. There is increasing evidence of iron accumulation during ageing, and altered iron levels in some specific brain regions in neurodegenerative disease patients have been reported.

Using graphite furnace atomic absorption spectrometry after microwave-assisted acid digestion of the samples, iron levels were determined in 14 different areas of the human brain [frontal cortex, superior and middle temporal, caudate nucleus, putamen, globus pallidus, cingulated gyrus, hippocampus, inferior parietal lobule, visual cortex of the occipital lobe, midbrain, pons (locus coeruleus), medulla and cerebellum (dentate nucleus)] of n=42 adult individuals (71±12 years old, range: 53-101 years old) with no known history or evidence of neurodegenerative, neurological or psychiatric disorders.

It was found that the iron distribution in the adult human brain is quite heterogeneous. The highest levels were found in the putamen (mean ± sd, range: 855±295 µg/g, 304-1628 µg/g) and globus pallidus (739±390 µg/g, 225-1870 µg/g), and the lowest levels were observed in the pons (98±43 µg/g, 11-253 µg/g) and medulla (56±25 µg/g, 13-115 µg/g).

Globally, iron levels proved to be age-related. The positive correlation between iron levels and age was most significant in the basal ganglia (caudate nucleus, putamen and globus pallidus).

Compared with the age-matched control group, altered iron levels were observed in specific brain areas of one Parkinson's disease patient (the basal ganglia) and two Alzheimer's disease patients (the hippocampus).

Keywords: human brain; iron levels; post-mortem analysis; ageing; neurodegenerative diseases

Introduction

According to the US National Institute of Aging and WHO's 2011 "Global Health and Aging" report [1] and as a result of longer life expectancy, chronic and degenerative diseases will become the most prevalent diseases worldwide over the next several decades.

The aetiology of neurodegenerative diseases (NDs) is multifactorial, and it is assumed to involve a complex interaction between (natural) ageing, genetic predisposition and environmental factors [2]. Disturbances in trace element (TE) homeostasis in specific areas of the brain [3, 4] have been identified as contributing to the development of many NDs.

Iron (Fe) is the most abundant transition metal in the human body and has many essential functions in the brain and nervous system, such as oxygen transport, mitochondrial respiration, protein and DNA synthesis, myelination, dendrite development and neurotransmitter biosynthesis [5, 6]. Interestingly, it has been observed that patients with NDs such as Parkinson's and Alzheimer's disease tend to accumulate Fe in their nervous system [7-12], suggesting a role for this transition metal in these disorders. Fe accumulation leads to excessive production of reactive oxygen species, protein, DNA and phospholipid oxidation and, ultimately, to structural and functional damage [3].

Despite the intensive research that has been conducted on the relationship between TE and ND, the evidence is still fragmentary, and the exact role of TE remains poorly understood. Its direct determination in human brain samples from both healthy individuals and patients with ND is crucial for a better understanding of the underlying disease mechanisms and the development of chelation therapies, but most of the current information about the relationship between TE and human brain functioning is based on animal studies [13, 14] or relies on determinations in cerebrospinal fluid,

blood or serum samples [13, 15-19]. Studies on TE levels in normal and pathological human brains are scarce, limited to a few brain areas [20-23] and/or involve a small number of subjects [24, 25].

Based on this background, the main goal of the present study was to directly quantify Fe levels in normal human brain tissue, extending the number of brain areas and the number of subjects studied, to evaluate a) the regional anatomic differences and b) age-related changes in Fe levels within the brain tissue. This type of information is indispensable for interpreting the data obtained from ND patients.

Materials and methods

Subjects

Brain samples were collected from men ($n=27$; 67 ± 11 years old) and women ($n=15$; 77 ± 12 years old) not registered in the Portuguese *National Registry of Refusal to Organ Donation* database and complying with all the current regulations regarding human tissue collection for scientific research purposes.

Samples were obtained from individuals submitted to forensic autopsy exams during the first semester of 2012 at the North Branch (Porto) of the Portuguese National Institute of Legal Medicine (INML). Individuals from each of the following age groups were studied: 50-60 ($n=10$), 60-70 ($n=10$), 70-80 ($n=10$), 80-90 ($n=9$) and ≥ 90 ($n=3$) years old. Inclusion criteria were a) the absence of a history of known neurodegenerative, neurological or psychiatric disorders, b) the absence of injuries involving the central nervous system (CNS), and c) macroscopically normal tissues.

Samples from two individuals with documented Alzheimer's disease (women, 73 and 85 years old) and one with Parkinson's disease (woman, 91 years old) were also collected.

Sample collection

Samples were collected by the pathologists at the INML following a standard protocol.

To prevent sample contamination, all materials in contact with the samples, including the stainless steel tools used by the pathologists, were previously decontaminated with a 5% (v/v) nitric acid solution prepared from concentrated ($\geq 69\%$) HNO₃ (Sigma-Aldrich, Germany) and thoroughly rinsed with ultrapure water (resistivity 18.2 M Ω .cm at 25 °C) produced by a Milli-Q water purification system (Millipore, USA).

After removing the brain from the cranium, the contaminating blood was washed away with ultrapure water. The meninges were removed with plastic tweezers, and the brain was washed again with ultrapure water to minimise sample contamination with blood or cerebrospinal fluid.

The brain areas studied were defined based on Paine and Lowe's recommendations for regions to be sampled when a neurodegenerative disease is suspected [26]. Using decontaminated plastic knives, tissue fragments (approximately 1 cm³) were collected from the following brain areas: the frontal cortex (1); superior (2A) and middle (2B) temporal gyri; basal ganglia, including the caudate nucleus (3A), putamen (3B) and globus pallidus (3C); cingulate gyrus (4); hippocampus (5); inferior parietal lobule (6); visual cortex of the occipital lobe (7); midbrain, including the *substantia nigra* at the level of the third nerve (8); pons (locus coeruleus) (9); medulla (10); and cerebellum (dentate nucleus) (11). Samples were stored in decontaminated polypropylene tubes (Sarstedt, Germany) at -4 °C until analysis.

Sample pre-treatment

After defrosting, the brain samples were thoroughly washed with ultrapure water and placed in a dry oven (Raypa, Spain) at 110 °C until constant weight (ca. 24 hours). Dried samples (ca. 100-500 mg) were weighed directly in the microwave digestion vessels, which had been previously decontaminated with 10% (v/v) HNO₃ and thoroughly rinsed with ultrapure water. Samples were digested using 2.5 mL of concentrated ($\geq 65\%$ m/m) HNO₃ (*TraceSELECT*[®], Fluka, France) and 1.0 mL of $\geq 30\%$ (v/v) H₂O₂ solution (*TraceSELECT*[®], Fluka, Germany). The sample digestion was performed in an MLS 1200 Mega microwave oven from Milestone (Italy) equipped with an HPR 1000/10 rotor using the following power (W)/time (min) program: 250/1, 0/2, 250/5, 400/5 and 600/5. After cooling, sample solutions were made up to 50 mL with ultrapure water and stored in closed propylene tubes at 4 °C until analysis.

Iron determination

Fe determination was performed using a PerkinElmer (Germany) model 4100 ZL atomic absorption spectrometer (longitudinal Zeeman background correction) equipped with a transversely heated graphite atomiser (THGA), end-capped graphite tubes with an integrated L'vov platform (PerkinElmer Part No. B3 000653) and an AS-70 autosampler. An Intensitron[™] (PerkinElmer) hollow cathode lamp was used as a light source ($\lambda=248.3$ nm). Argon of 99.9999% purity (Gasin, Portugal) was used as an inert gas. A commercial magnesium nitrate solution (ref. 63043; Fluka, Switzerland) was used to prepare the matrix modifier solution [5 μ L=15 μ g Mg(NO₃)₂]. The instrumental conditions and graphite furnace program used are summarised in Table 1.

Table 1

Instrument settings and graphite furnace program for Fe determination.

<i>Parameter</i>	<i>Value</i>		
Wavelength	248.3 nm		
Slit width	0.2 nm		
Lamp current	30 mA		
Inert gas	Argon		
Flow rate	250 mL/min (0 at the atomization step)		
Background correction	Longitudinal Zeeman-effect		
Sample injection volume	15 μ L		
Matrix modifier injection volume	4 μ L		
Measurement mode	Integrated absorbance (A.s)		
Integration time	5 s		
Baseline Offset Correction (BOC)	2 s		
<i>Graphite furnace program</i>			
Step	Temp. ($^{\circ}$C)	Ramp (s)	Hold (s)
Dry	110	1	30
Dry	130	15	30
Pyrolysis	1400	10	20
Atomization	2100	0	5
Cleaning	2450	1	3

Injection temperature: 20 $^{\circ}$ C.

The sample digests were diluted (100-fold) with 0.2% (v/v) HNO₃ solution before injection into the graphite tube. Sample solutions were measured in duplicate. For results with RSD \geq 10%, an additional two injections were performed.

Calibration standards were also prepared by diluting a commercial multi-element standard solution (PerkinElmer Pure; Part No. N9300244) with 0.2% (v/v) HNO₃ solution. Calibration curves were obtained with six calibration standards with concentrations ranging from 10 to 60 μ g/L.

Analytical quality control

Because human brain tissue is not available as a certified reference material (CRM) for Fe determination, DOLT-4 Dogfish Liver and DORM-3 Fish Protein CRMs for Trace Metals (National Research Council, Canada) were used for analytical quality control purposes. CRMs were subjected to the same sample pre-treatment and employed at the same concentration levels as the sample

solutions. The values obtained were within the analytical uncertainty of the certified values (Table 2).

Table 2

Results ($\mu\text{g/g}$) obtained in the determination of Fe in DOLT-4 and DORM-3 Certified Reference Materials.

DOLT-4		DORM-3	
Reference value*	Experimental value	Reference value*	Experimental value
1833 \pm 75	1934 \pm 63	347 \pm 20	349 \pm 30

*Results obtained using ICP-MS and ICP-AES.

For contamination control of the microwave-assisted acid digestion procedure, a sample blank was included in each digestion run (10 samples). In total, 77 sample blanks were run, and the mean Fe content obtained (0.37 $\mu\text{g/L}$) was subtracted from the sample values.

Statistical evaluation

The descriptive statistical parameters (mean and standard deviation) were calculated using Microsoft Office Excel 2010 (Microsoft Co., USA). The means were compared by an unpaired Student's t-test at a 0.05 significance level using GraphPad Prism 5 (GraphPad Software Inc., USA).

Results and discussion

Regional distribution of Fe

The results ($\mu\text{g/g}$, dry weight) for Fe determination in each of the 14 different brain regions studied are summarised in Table 3.

Table 3

Fe levels (mean±sd, µg/g) in 14 different regions of human brain (see footnote) of non-diseased individuals (n = 42; 71 ± 12 years old) according to age group.

Brain regions*	All individuals	Age groups				
		[50-59] (n=10)	[60-69] (n=10)	[70-79] (n=10)	[80-89] (n=9)	≥90 (n=3)
1	275±65	241±41	280±43	315±72	244±51	306±135
2A	289±67	269±55	289±48	312±70	281±91	306±86
2B	287±65	269±55	276±57	308±77	256±36	382±81
3A	599±271	533±268	532±249	607±259	743±360	398±215
3B	855±295	777±267	762±133	848±351	1155±363	976±286
3C	739±390	577±266	759±371	593±269	1062±526	735±331
4	226±64	194±73	231±53	246±57	226±79	243±43
5	239±54	239±55	239±66	237±52	246±64	227±1
6	288±68	279±67	283±77	305±62	288±87	272±21
7	332±113	312±82	308±79	396±170	307±107	347±69
8	293±106	257±89	321±119	277±101	327±118	271±133
9	98±43	87±30	98±40	99±40	116±70	85±45
10	56±25	48±19	56±26	71±21	45±10	48±43
11	283±114	244±135	287±129	299±98	305±103	288±139
Mean of studied regions	347±63	309±56	335±52	346±47	402±7	358±22

*1-Frontal cortex; 2A-Superior temporal gyrus; 2B-Middle temporal gyrus; 3A-Caudate nucleus, 3B-Putamen; 3C-Globus pallidus; 4-Cingulated gyrus; 5-Hippocampus; 6-Inferior parietal lobule; 7-Visual cortex of the occipital lobe; 8-Midbrain, 9-Pons 10-Medulla; 11-Cerebellum.

The results showed that the Fe distribution in an adult human brain is quite heterogeneous. Regardless of age group, the highest levels were found in the basal ganglia (putamen: 328-1628 µg/g, globus pallidus: 225-1870 µg/g, caudate nucleus: 199-1477 µg/g) and the lowest in the pons (11-253 µg/g) and medulla (13-115 µg/g). Previously published data [3, 25] also suggested that normal Fe content within the brain varies significantly by region, with the highest levels being found in the *substantia nigra*, basal ganglia, hippocampus and subcortical brain regions. House et al. [23] evaluated the Fe content in the frontal, temporal, parietal and occipital lobes of 60 elderly individuals (70-103 years old) and found an overall tissue content of *ca.* 290 µg/g (range: 112-8350 µg/g) with

significant differences ($p < 0.05$) between the frontal and occipital as well as the frontal and parietal lobes. In our study, no difference was found between the latter two regions.

The basal ganglia are the largest component of the extrapyramidal system and are considered part of the motor system [27]. However, the cerebellum, another brain region involved in motor coordination [27], presented much lower Fe levels (109-520 $\mu\text{g/g}$). The reason for this selective distribution of Fe in distinct areas related to motor functions is unknown. Increased oxygen demand cannot provide a full explanation because other brain regions with intense metabolic activity do not show comparable Fe levels [28].

Age-related changes

Globally, i.e., considering the mean value of the results obtained for the 14 regions, a tendency for an age-related increase in Fe levels in brain was found (Fig. 1a).

This tendency was particularly significant in some specific areas. The most significant direct correlation between Fe levels and age was found in the caudate nucleus, putamen and globus pallidus (Fig. 1b-d).

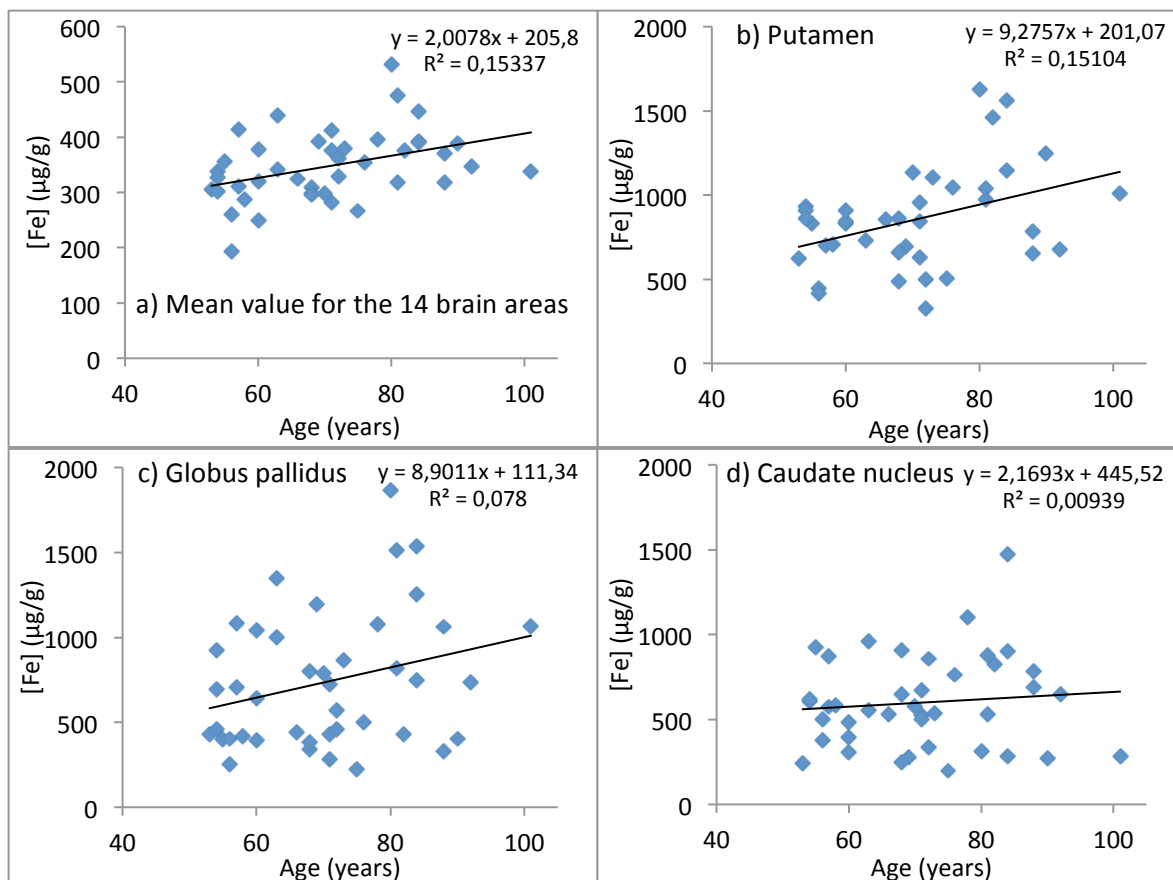


Fig. 1. Relationship between Fe brain levels ($\mu\text{g/g}$, dry weight) and age (years): mean value of the different 14 brain regions for each individual ($n = 42$) (a) and Fe levels in putamen (b), globus pallidus (c) and caudate nucleus (d).

Several other post-mortem and *in vivo* studies also found an increase in Fe levels with age in the subcortical and some cortical grey matter regions in healthy individuals. Xu et al. [29] reported an age-related Fe accumulation in the putamen from 22 years to over 70 years, reaching maximal values in approximately the sixth decade. The authors reported that the Fe levels in the globus pallidus and caudate nucleus remained unchanged with age, but our results show a positive correlation in these regions. Aquino et al. [30] also report an exponential increase in Fe with increasing age in all the basal ganglia of neurologically healthy individuals ($n=80$, 1-80 years old). Adisetiyo et al. [31] found

significantly lower Fe levels in adolescents (n=16, 13-18 years old) than adults (n=10, 21-44 years old) in the caudate nucleus, putamen and globus pallidus.

Hebbrecht et al. [22] studied five regions (the basal ganglia, cerebral cortex, cerebral white matter, brainstem and cerebellar cortex) of the human brain (n=18, 7-79 years old) and found a strong correlation between Fe levels and age in all these areas. Bilgic et al. [32] also found that elderly people had significantly higher Fe levels in the striatal regions of the putamen, caudate nucleus, globus pallidus and *substantia nigra* than younger people. Zecca et al. [33] evaluated Fe content in the *substantia nigra* during ageing and found a smooth increase within the considered age interval (16-90 years old).

In a recent study by Exley et al. [34], no statistically significant correlation between brain Fe levels and age was found for a group of 60 elderly individuals (70-103 years old), which may indicate that this trend disappears in very old people.

When individuals were grouped by gender, quite similar Fe levels were observed (346 ± 36 $\mu\text{g/g}$ for women *versus* 348 ± 73 $\mu\text{g/g}$ for men; $p=0.976$), with a lower inter-individual variability in women. Xu et al. [28] also reported no significant gender-related differences in brain Fe levels. This is an interesting finding because it is well known that women have lower whole-body Fe levels than men; therefore, a lower brain level could also be expected. The female subgroup (n=15) had a higher mean age (77 ± 12 years old) than the male subgroup (n=27; 67 ± 11 years old), but this is not a sufficient explanation for the similarity in brain Fe levels because our results also showed that the positive correlation between Fe levels and age is not observed in women (Fig. 2).

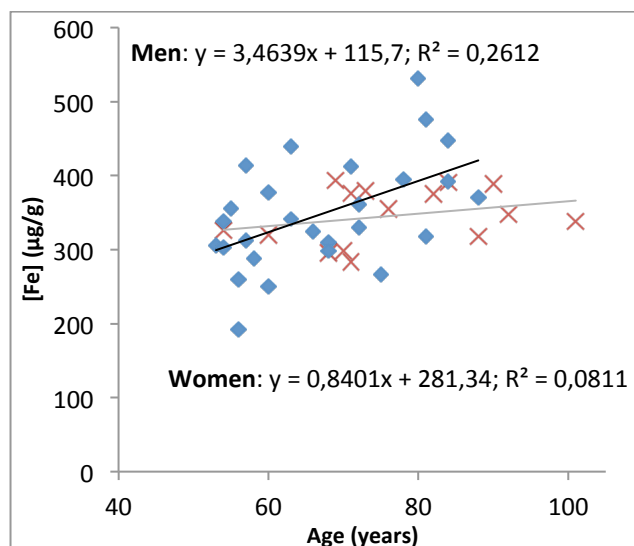


Fig. 2. Relationship between brain Fe levels ($\mu\text{g/g}$) and age (years) in men (\blacklozenge) and women (\times).

Disease-related changes

Abnormally high brain levels of Fe have been reported in several NDs, including Parkinson's disease and Alzheimer's disease [35-39]. Because brain tissue is rich in oxygen and fatty acids, elevated Fe levels provides a perfect environment for oxidative stress and severe tissue damage [37].

When compared with non-diseased subjects of the same age group, the brain Fe levels of the two Alzheimer's disease patients were elevated in the caudate nucleus (by factors of 1.9 and 1.7) and the hippocampus (by factors of 1.3 and 2.1), the main brain region associated with memory and spatial navigation [40]. The Parkinson's disease patient presented more pronounced differences in the basal ganglia (the brain regions with the highest Fe levels in non-diseased individuals): the Fe levels were elevated in the caudate nucleus (by a factor of 2.3) and the globus pallidus (by a factor of 2.4), which are both associated with motor functions [26].

Conclusion

This study, which directly determined Fe levels in 14 different brain regions in 42 non-diseased individuals, provides further evidence of the quite heterogeneous distribution of Fe in the human brain.

Globally (i.e., considering the mean value of the results obtained for the 14 regions in each individual), a significant age-related tendency for increased brain Fe levels was found. The basal ganglia were the brain regions where this direct correlation was most significant, whereas the correlation was almost absent in several other brain regions.

No significant gender-related difference was observed in brain Fe levels. However, the direct correlation between total Fe levels and age was not observed in women; their brain Fe levels showed remarkable consistency in the age interval studied.

This work provides a comprehensive and updated background for Fe levels in non-diseased human brains. It may be a significant contribution for defining “normal” Fe levels, which would allow future interpretations of results obtained in studies of patients affected by ND. This work also highlights the need for clearly defining the brain areas that are effectively studied and the age of the patients when performing such studies.

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