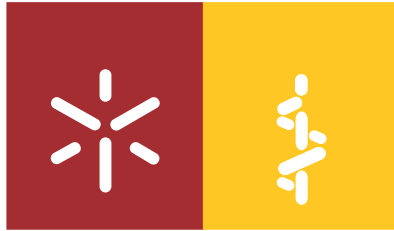**Universidade do Minho**
Escola de Ciências da Saúde

José Carlos Leitão Portugal Nunes

Cognitive and physical performance correlates of low iron status

julho de 2014

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Cognitive and physical performance correlates of low iron status

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Trabalho efetuado sob a orientação da
**Professora Doutora Joana Almeida Santos
Pacheco Palha**

e coorientação da
Doutora Nadine Correia Santos

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Assinatura: _____

"Would you tell me, please, which way I ought to go from here?''

''That depends a good deal on where you want to get to,'' said the Cat.

''I don't much care where –'' said Alice.

''Then it doesn't matter which way you go,'' said the Cat.

''– so long as I get *somewhere*,'' Alice added as an explanation.

''Oh, you're sure to do that,'' said the Cat, ''if you only walk long enough.''

''But I don't want to go among mad people,'' Alice remarked.

''Oh, you can't help that,'' said the Cat: ''We're all mad here. I'm mad. You're mad.''

''How do you know I'm mad?'' said Alice.

''You must be,'' said the Cat, ''otherwise you wouldn't have come here.''

Lewis Carroll's novel, *Alice's Adventures in Wonderland*

A toda a minha família, mas em especial ao mais recente membro, a minha sobrinha Diana, e ao seu mais antigo elemento, a minha Avó.

À minha Irmã por ser um exemplo de força e carácter e um dos meus mais sólidos pilares.

Aos meus Pais.

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À Margarida por ser sempre um ombro amigo, por estar sempre presente, por olhar para a minha cara e perceber o meu estado de espirito. Por me colocar sempre mais e mais desafios. Por tantas coisas que tenho para agradecer e porque tenho tão poucas palavras para o fazer, resta-me apenas dizer que agradeço por tudo. Que venham muitos anos como estes últimos 2 anos e meio de preferência acompanhados com muita Erdinger Weissbier.

Abstract

Iron deficiency is the most prevalent nutrient deficiency affecting all age groups worldwide. Deleterious alterations on cognition, psychological morbidity and physical performance have been observed in several reports; however, the main body of literature focuses on infants, children, adolescents or women of childbearing age. These groups are considered to be at high risk due to physiologic conditions. Nonetheless, several pathological conditions are also risk factors for iron deficiency, with many of them highly prevalent in older individuals. In a growing older society, considering that older individuals are at greater risk of cognitive decline, neuropsychological morbidity, decrements in physical performance and impaired functional ability, the study of the factors influencing these outcomes are of utmost importance. Still, despite of all the evidence pointing for a role of iron deficiency in cognition, mood and physical functional ability, there is a worrying small amount of research in older individuals.

In this work, by first using a cross-sectional analysis, we investigated the association of low iron status with cognitive performance, neuropsychological morbidity and physical functional ability in a cohort of older individuals (n=151). Next, using a quasi-experimental study design (n=12, intervention; n=10, non-intervention), namely intervention via an iron-fortified fruit–based dessert, we addressed if low dosage iron fortification of foods is feasible and effective in altering (and/or correcting for) the effects of low iron status.

In order to reduce the number of multiple comparisons, principal component analysis of cognitive, psychological, physical variables and iron biomarkers was performed and the obtained dimensions used for analysis. We observed that the storage [body iron, soluble serum transferrin receptor (sTFR), ratio of sTFR to the logarithmic value of ferritin (sTFR – Log(FT)) index and ferritin (FT)] and erythropoiesis [red cells blood Count (RBC), hemoglobin and hematocrit] dimensions were significant predictors of the memory dimension [selective reminding test (SRT) - consistent long term retrieval (CLTR), - long term storage (LTS) and - delayed recall (DR) and the Consortium to establish a registry for Alzheimer's disease (CERAD) (total hits and DR)], along with the interaction of storage and nutritional status. The geriatric depression scale (GDS) score was predicted by the transport [serum iron (Fe) and transferrin saturation (TF sat.)], transport saturation [transferrin (TF) and total iron binding capacity (TIBC)] and erythropoiesis dimensions. The functional tiredness (mobility-, lower limb- and upperlimb-tiredness) dimension was predicted by the storage, transport, red cells composition [mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW)] and erythropoiesis dimensions. After 12 weeks (+/- 2 weeks) of intervention, the daily consumption of an iron fortified dessert was associated with an improvement of the total and

sub-scores of performance-oriented mobility assessment, along with hand grip strength and lower limb tiredness.

Our observations indicate that lower iron status is associated with poorer memory ability, depressive mood and functional tiredness from activities of daily living. Furthermore, results indicate that the physical negative effects of low iron status seem to be recovered by iron supplementation, highlighting the importance of prevention. Identification of molecular bases of the associations here reported is paramount and further research is needed.

Resumo

A deficiência de ferro é o *deficit* nutricional mais prevalente em todo o mundo, afetando todas as faixas etárias. Vários estudos têm demonstrado um impacto negativo da deficiência de ferro sobre a cognição, a morbidade psicológica e desempenho físico; no entanto, os referidos estudos debruçam-se essencialmente em crianças, adolescentes ou mulheres em idade fértil, nos quais várias condições fisiológicas concorrem para que estes grupos sejam considerados de alto risco. No entanto não devemos descurar, igualmente, que várias condições patológicas são fatores de risco para deficiência de ferro, sendo muitas delas altamente prevalentes em idosos. Numa sociedade cada vez mais envelhecida, e onde os indivíduos mais velhos apresentam risco acrescido de declínio cognitivo, morbidade neuropsicologia, decréscimos no desempenho físico e capacidade funcional comprometida, o estudo dos fatores que influenciam estas consequências do envelhecimento são de extrema importância. Apesar de todas as evidências apontarem para um papel da deficiência de ferro na cognição, humor e capacidade física funcional, a investigação em idosos é escassa.

Neste trabalho, utilizando uma análise transversal, investigámos as associações de baixos níveis de ferro com o desempenho cognitivo, a morbidade neuropsicológica e a capacidade funcional física em idosos. Através de um estudo quasi-experimental, investigamos, ainda, se a fortificação de alimentos com pequenas doses de ferro é viável e eficaz para melhorar a capacidade cognitiva, o humor e a condição física.

Com o objetivo de reduzir o número de comparações múltiplas foi realizada a análise dos componentes principais das variáveis cognitivas, psicológicas, físicas e dos biomarcadores de ferro e os componentes obtidos utilizados para análise estatística. Observou-se que os componentes armazenamento [ferro corporal, receptor solúvel da transferrina (sTFR), indice do rácio do sTFR para a transformação logarítmica da ferritina (sTFR – Log(FT)) e ferritina (FT)] e eritropoiese [eritrócitos (RBC), hemoglobina e hematócrito], bem como a interação do componente armazenamento com estado nutricional, foram preditores significativos do componente memória [teste de memoria selectiva (SRT) – evocação da memória a longo prazo (CLTR), - armazenamento na memória a longo prazo (LTS) e – evocação tardia (DR); e o Consórcio para estabelecer um registro para a doença de Alzheimer (CERAD) (total de respostas certas e DR)]. O valor da escala de depressão geriátrica (GDS) foi previsto pelos componentes transporte [ferro sérico (Fe) e saturação da transferrina (TF sat.)], saturação do transporte [transferrina (TF) e capacidade total de ligação do ferro (TIBC)] e da eritropoiese. Os componentes armazenamento, transporte, composição células vermelhas [volume corpuscular médio (MCV), hemoglobina corpuscular média (MCH), concentração de hemoglobina

corpuscular média (MCHC) e a anisocitose (RDW)] e eritropoiese foram preditores significativos do componente obtido para o cansaço associado às atividades funcionais diárias. Observámos, ainda, que após 12 semanas (+ / - 2 semanas), o consumo diário de uma sobremesa fortificada em ferro se associa a uma melhoria no total e subtotais de avaliação da mobilidade orientada para o desempenho, juntamente com força de preensão manual e menor cansaço dos membros inferiores.

Estes resultados indicam que os níveis de ferro estão associados a menor memória, a humor depressivo e a cansaço funcional nas atividades da vida diária. Além disso, os resultados sugerem que os efeitos negativos de baixo nível de ferro a nível físico melhoram com a suplementação de ferro, destacando a importância da prevenção do declínio nos níveis de ferro. Importa no futuro a confirmação destes resultados em estudos de base populacional, assim como a identificação das bases moleculares que lhes estão subjacentes.

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Abbreviations list

6MTW	6 meter timed walk
ABCB7	ATP-binding cassette, subfamily B, member 7
ACD	Anemia of chronic disease
ACER	Addenbrooke's cognitive examination-revised
ANCOVA	Analysis of covariance
ATP	Adenosine triphosphate
BAI	Beck anxiety inventory
BDI	Beck depression inventory
BF	Body fat
BIA	Bioelectrical impedance analysis
BMI	Body mass index
BNT-15	Boston naming test 15-item version
C	Colors
CCA-B	Clinical academic center – Braga
CERAD	Consortium to establish a registry for Alzheimer's disease
circ.	Circumference
CLTR	Consistent long term retrieval
Cog.	Cognition
COWAT-FAS	Controlled oral word association test F-A-S
CP	Ceruloplasmin
DALY	Disability adjusted life years
DASS-21	Depression, anxiety and stress scales – 21 items
DCYTB	Duodenal cytochrome b
df	Degrees of freedom
DMT1	Divalent metal transporter 1
DR	Delayed recall
DS	Digits spam
FAO	Food and Agriculture Organization of the United Nations
Fe	Serum iron
Fe^{2+}	Ferrous iron
Fe^{3+}	Ferric iron
Fe-S	Iron-sulfur
FLVCR	Feline leukemia virus subgroup C receptor
FPN	Ferropotin
FT	Ferritin
GDS	Geriatric depression scale
H. pylori	*Helicobacter pylori*
HCP1	Heme carrier protein 1
HIF	Hypoxia inducible factor
HO	Heme oxigenase
HP	Hephaestin
hsCRP	High sensitive C-reactive protein
ID	Iron deficiency

IDA	Iron deficiency anemia
IL-6	Interleukin-6
IRE	Iron responsive element
IRP	Iron responsive protein
Log	Base ten logarithm
LTS	Long term storage
MCH	Mean cell hemoglobin
MCHC	Mean cell hemoglobin concentration
MCV	Mean corpuscular volume
MMSE	Mini mental state examination
MNA	Mini nutritional assessment
MOCA	Montreal cognitive assessment
Na(Fe^{3+})EDTA	Ethylenediaminetetraacetic acid ferric sodium salt
NHANES	National health and nutrition examination survey
NSAID	Nonsteroidal anti-inflammatory drug
OR	Odds ratio
PCA	Principal component analysis
PCFT	Proton coupled folate transporter
POMA	Performance oriented mobility assessment
PSS	Perceived stress scale
QoFA	Questionnaire of functional ability
RBC	Red cells blood Count
RDW	Red cell distribution width
SD	Standard deviation
SE	Standard error
SOP	Standard operating procedures
SRT	Selective reminding test
STEAP	Six transmembrane epithelial antigen of the prostate
sTFr	Soluble serum transferrin receptor
sTFr-LogFT	Ratio of sTFR to the logarithmic value of ferritin
Supplem.	Supplemented
TF	Transferrin
TF sat.	Transferrin saturation
TFR	Transferrin receptor
TIBC	Total iron binding capacity
W	Words
W&C	Words/colors
WAIS III	Wechsler adult intelligence test
WHO	World health organization
yrs	Years

1. Introduction

Iron is essential for several biochemical/biological processes. Namely, it is a component and cofactor of various enzymes, participating in oxygen transport and storage, mitochondrial electron transport, catecholamine metabolism and neurotransmitters and DNA synthesis, among others (J. L. Beard, Connor, & Jones, 1993; Hill, 1985; IOM, 2001; M. B. Youdim & Green, 1978). In normal conditions, healthy adults have approximately 35 to 45 mg of iron per kilogram of body weight (premenopausal females have lower iron stores due to recurrent menstrual blood losses), being more than two thirds incorporated in hemoglobin (Nancy C. Andrews, 1999). Imbalance in iron homeostasis, both excess and deficiency, are deleterious to human health and are associated with conditions such as hemochromatosis, neurodegenerative disorders (i.e. Parkinson and Alzheimer diseases), type II diabetes and anemia (Iron Deficiency Anemia – IDA) (Hentze, Muckenthaler, & Andrews, 2004; Jiang et al., 2004; Zecca, Youdim, Riederer, Connor, & Crichton, 2004; Zhao et al., 2012).

Paradoxically, although iron is one of the most abundant elements on the planet, iron deficiency is the most common nutritional deficiency (Boccio & Iyengar, 2003). While several causes can be present in the etiology of the disorder, it can, in general, be explained as an imbalance between iron intake, absorption and losses (Cook, 2005; Zimmermann & Hurrell, 2007). The inability to maintain, for a long period of time, adequate plasma iron levels and/or body iron stores, leads to iron deficiency (ID) anemia (IDA) (De Domenico, McVey Ward, & Kaplan, 2008), the most common hematological disorder (McLean, Cogswell, Egli, Wojdyla, & de Benoist, 2009; Mukhopadhyay & Mohanaruban, 2002). In fact, the World Health Organization (WHO) estimates that, worldwide, more individuals have IDA than any other health problem (Mathers, Fat, Boerma, & Organization, 2008; McLean et al., 2009). Although all age groups are vulnerable to ID, infants, adolescents, women of childbearing age or pregnant, and middle-aged/older individuals (defined as age ≥ 60 years) are particularly susceptible (De Benoist, Cogswell, Egli, & McLean, 2008; McLean et al., 2009; WHO, 2001). In adults, ID and IDA can result or be associated with a wide range of adverse effects, including: fatigue, reduced work performance, diminished exercise capacity, impaired thermoregulation, immune dysfunction, gastrointestinal disturbances, and neurocognitive impairment (Susan F. Clark, 2008; Haas & Brownlie, 2001; Zimmermann & Hurrell, 2007).

Of particular concern, the raise of life expectancy in the last century has led to an increase of the aged population worldwide (Christensen, Doblhammer, Rau, & Vaupel, 2009). Specifically, in the last 160 years women's life expectancy has increased 3 months per year in an

almost linear trend, with the same trend observed for men, albeit at a slower pace (Oeppen & Vaupel, 2002). 'Normal' healthy aging is associated with a degree of cognitive decline, termed "age-related cognitive decline/aging" (defined as no dementia, mild cognitive impairment or other specific cognitive decline-associated syndromes/diseases), as well as with physical challenges and decrements (Beddington et al., 2008; Deary et al., 2009; Guralnik, Fried, & Salive, 1996; Janssen, Heymsfield, & Ross, 2002). Despite being indisputable that life expectancy increase should be celebrated, this current demographic and societal phenomenon will result in an increasing number of older individuals with various age-associated health concerns/problems and pathologies (including, for example, cancer, fractured hips, strokes, dementia), which may occur concurrently (that is, co- or multi-morbidities) (Rechel et al., 2013; The, 2012). Furthermore, aged individuals are also the largest consumers of prescribed drugs or medication (Qato et al., 2008), with age-associated disease burden and medication consumption accounting for significant health care needs, which is reflected by an increase in the expenditure of the health and welfare systems of nations (Rechel et al., 2013). Of relevant note, several morbidities and therapeutic drugs that are highly prevalent in older individuals are also possible causes of ID (see 1.2.1 – From basics to clinics) (Susan F. Clark, 2008).

Taken together, aging and ID can be deleterious for health and wellbeing, particularly in cognition and physical ability. Notably, however, extremely little focused research has been conducted in this population strata. A systematic review and meta-analysis found an increased risk of incident dementia in anemic individuals; however, the type of anemia was not addressed (Peters et al., 2008). Although anemia is used as an indicator of ID and the terms anemia, ID and IDA are used interchangeably, it should be noticed that anemia can also be caused by vitamin B_{12} deficiency, which is a well-known cause of dementia (Reynolds, 2006; WHO, 2001). Furthermore, physical functional ability in the elder has been associated with anemia (Chaves, 2008; Denny, Kuchibhatla, & Cohen, 2006; Penninx et al., 2003; Penninx et al., 2004; Mya Thein et al., 2009). Still, to the best of our knowledge no study has addressed associations with ID.

The growing older population and the potential reversibility, amelioration or prevention of the delirious effects of ID in cognition and physical functionality dictate the need of studies that multi-disciplinarily address and explore the association between iron status, cognitive ability and physical functional performance in middle-aged/older individuals.

1.1. Iron homeostasis

The physiology of iron metabolism has been known for more than a half a century, mostly by means of human and animal studies using iron isotopes. More recently, the discoveries of key molecules that are involved in intestinal iron absorption allowed a better understanding of this process at the molecular level (Frazer & Anderson, 2005; T. Ganz, 2008). Iron is a nutrient classified as an essential trace element with the ability to easily gain and loose electrons (inter-conversion between ferric [Fe^{3+}] and ferrous [Fe^{2+}] forms) (De Domenico et al., 2008; T. Ganz, 2008; Hentze et al., 2004). This chemical property of iron makes it an useful component of oxygen binding molecules, cytochromes and non-heme enzymes, largely explaining its biological importance, but also underlying the reasons for its deleterious and toxic effects when in excess (Hentze et al., 2004). Iron can catalyze a "fenton-type" redox reaction where the ferrous form interacts with hydrogen peroxide or lipid peroxidases originating free radicals (i.e. superoxide anions and the hydroxyl radical) that 'attack' and damage cellular membranes, nucleic acids and proteins (Nancy C. Andrews, 1999; Hentze et al., 2004). In humans, there is no known physiological regulated form to actively excrete iron, and since both iron overload and deficiency lead to several disorders, iron homeostasis must be tightly regulated via absorption and storage mechanisms (Nancy C. Andrews, 1999; De Domenico et al., 2008; Zimmermann & Hurrell, 2007). Daily, 1 to 2 mg of iron is absorbed from dietary sources, with similar amounts lost by epithelial cell shedding (skin, gastrointestinal and urinary tract cells) and fluids loses (menstruation, minor bleeding, tears and sweat) (T. Ganz, 2008; Miret, Simpson, & McKie, 2003). Despite of the small amounts that renew (and/or 'recycle') the iron pool, the erythron (all the erythrocyte, their precursors and organs involved in their production) has daily requirements of 20 to 30 mg (Nancy C. Andrews, 1999; Miret et al., 2003).

1.1.1. Iron absorption and metabolism

As mentioned, although 1 to 2 mg of iron are absorbed by enterocytes each day to compensate the losses, these amounts only represent a small portion of the body iron daily needs. The mean dietary intake of iron for an adult ranges from 12 to 18 mg/day, which is sufficient to meet the dietary reference allowance (8 mg/day for men and post-menopausal women and 18mg/day for pre-menopausal women) (IOM, 2001). Dietary iron can be divided in

two components: (i) heme iron (protoporfirin IX – from hemeproteins) and (ii) nonheme iron (dietary ferritin and iron salts and chelates). These have distinct bioavailabilities. Heme iron is found mainly in meat food sources and corresponds to up to 15% of the dietary iron, the remaining is nonheme iron which is found mainly in vegetable sources (Hallberg, 2001). Heme and nonheme iron are absorbed in the small intestine, where a gradient for absorption occurs, with higher rate at the duodenum and decreasing in the jejunum and in the ileum (De Domenico et al., 2008; Gibson, 2005; Miret et al., 2003). The enterocytes (polarized intestinal epithelial cells) are responsible for all iron absorption (Nancy C. Andrews, 1999). These cells, which derive from stem cells in the intestinal crypts (crypts of Lieberkuhn) and migrate up the villus, are characterized by an apical side (presenting the brush-border) and a basolateral side that are in contact with the intestinal lumen and the blood stream, respectively (Fuqua, Vulpe, & Anderson, 2012). Even though not all the processes involved in the intestinal iron absorption are clearly understood, it is know that absorption occurs through the transport across the apical membrane, translocation across the cytosol and release to the circulation through the basolateral membrane (De Domenico et al., 2008; Han, 2011). Heme and nonheme iron are absorbed by different mechanisms in an independent and not mutually exclusive manner (Nancy C. Andrews, 1999; Han, 2011; West & Oates, 2008), with much of what is known about iron absorption being limited to nonheme iron, despite of heme iron being far more efficiently absorbed and having outmost importance from the nutritional point of view (Miret et al., 2003; Wienk, Marx, & Beynen, 1999).

Evidence sustains two hypotheses for heme iron absorption: (i) iron binds to a receptor in the brush-border membrane of duodenal enterocytes and is translocated by endocitosys across the apical membrane, and (ii) iron is transported from the intestinal lumen to the cytoplasm of the enterocyte through the heme carrier protein 1/proton coupled folate transporter (HCP1/PCFT), whose physiological relevance is unclear (Figure 1) (Shayeghi et al., 2005; West & Oates, 2008; Zimmermann & Hurrell, 2007). Evidence also indicates that heme iron can, as well, be absorbed by receptor-mediated endocytosis, but a high-affinity receptor for heme iron has yet to be identified (Fuqua et al., 2012; West & Oates, 2008). Morphological studies using electron microscopy report the appearance of secondary lysosomes containing heme, in rats and dogs, upon administration of heme or hemoglobin close to the duodenal loops (West & Oates, 2008).

Absorption of dietary ferritin, a protein that binds inorganic iron, has begun to be recently more studied than all other nutritional forms of iron (E. C. Theil, 2004; Elizabeth C Theil, 2011). However, although there is some evidence that it is taken into the enterocyte by a phytate-resistant clathrin (receptor)-dependent endocytosis, mediated by a not yet identified high affinity receptor, the precise mechanism is unknown (Fuqua et al., 2012; Elizabeth C Theil, 2011). With exception for the dietary ferritin, nonheme iron is transported across the brush-border membrane via the divalent metal transporter 1 (DMT1) (Nancy C. Andrews, 1999; De Domenico et al., 2008; Miret et al., 2003; Zhang & Enns, 2009a, 2009b). The DMT1 co-transports hydrogen and iron and, as the name indicates, iron must be in its ferrous state (Fe^{2+}) (De Domenico et al., 2008; Mackenzie & Garrick, 2005; Zhang & Enns, 2009b). Most of the dietary iron is present in the intestinal lumen in the ferric form (Fe^{3+}), so it must be reduced prior to transportion by a brush-border ferric reductase. The duodenal cytochrome b (DCYTB) is a major reducing agent, but other reductases, such as the six transmembrane epithelial antigen of the prostate 2 (STEAP2), and ascorbic acid, also contribute (Nancy C. Andrews & Schmidt, 2007; Fuqua et al., 2012; T. Ganz, 2008; Han, 2011; Hentze et al., 2004; Zimmermann & Hurrell, 2007). Ascorbic acid can exert the effect of a reducing agent in itself or hypothetically act as a cofactor of the DCYTB (Mackenzie & Garrick, 2005; McKie et al., 2001).

Intestinal nonheme iron absorption can be affected by several factors that range from the physiologic status of the individual (discussed further ahead) to dietary factors. In fact, although it is now possible to shed light and/or explore several of the luminal aspects that affect iron absorption, the establishment of a quantitative model for it remains complex (Hallberg, 2001; Miret et al., 2003). Once iron is released from food components, ferric iron will remain soluble as long as the environment favors an acidic pH, which is not a problem in the stomach but can be at the intestinal lumen. The acidic microenvironment of the duodenal surface, along with the cell surface reductase activity from the DCYTB will maintain significant levels of soluble and ferrous iron (Miret et al., 2003). Furthermore, several luminal reactions can occur with food components or secreted molecules that will enhance or inhibit nonheme iron absorption. From the dietary components that affect iron absorption citric acid, ascorbic acid and some amino acids (e.g.: cysteine) will favor iron absorption either by maintaining iron soluble (citric acid) or by reducing ferric iron to ferrous iron (ascorbic acid and cysteine). On the contrary, the presence of phytate, polyphenols and tannic acid will result in the formation of complexes with iron (booth ferric and ferrous iron) and inhibit its absorption (Han, 2011). Calcium, copper and lead, due to absorptive

competition, are also involved in the inhibition of nonheme iron (Nancy C. Andrews, 1999; Pasricha et al., 2010). Furthermore, gastroferrin, a stomach glycoprotein, is probably an important iron absorption regulator by the formation of iron complexes; although, the direction of the regulation is still debatable (Miret et al., 2003). On the other hand, heme iron is far more bioavailable than nonheme iron and therefore its absorption is less affected by the external factors here addressed for nonheme iron (Han, 2011).
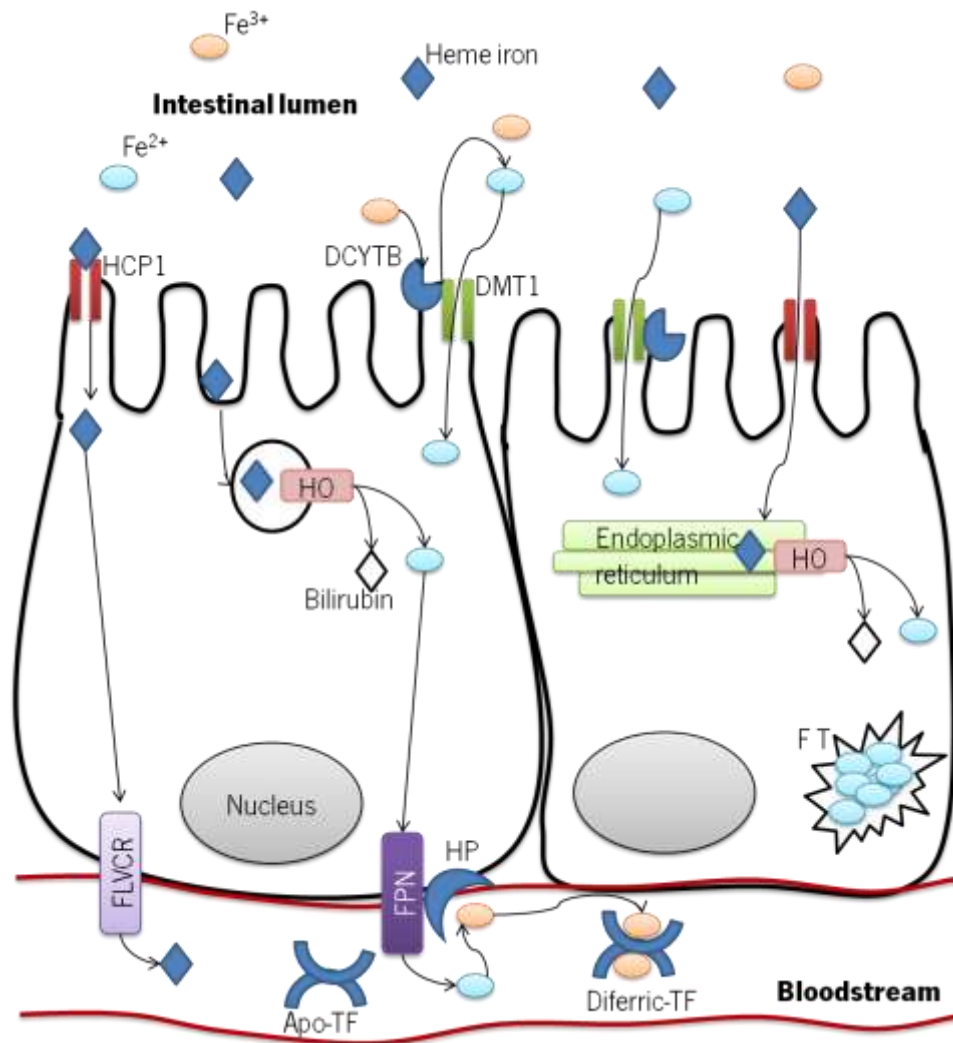
Figure 1 - Summary diagram of the established and putative iron absorption pathways in the intestinal enterocyte.

After entering the enterocyte, iron has two possible fates: (i) stored in ferritin (FT), or (ii) exported and reach circulation (Nancy C. Andrews, 1999). Again, there is some lack of knowledge regarding heme iron metabolism. It is known that it is absorbed, appearing in

membrane bound vesicles where it is degraded by heme oxigenase (HO), splitting it into bilirubin and ferrous iron. These will then enter the labile iron pool in the enterocyte, although the mechanism by which iron leaves the vesicle is unknown (Shayeghi et al., 2005; West & Oates, 2008). In support, it has been shown that 90% of the iron from radio-labeled hemoglobin administered via enteral route is recovered after 3 hours from the portal circulation as nonheme iron (West & Oates, 2008). This mechanism is plausible for the heme iron that enters the enterocyte by endocytosis. The heme iron that is thought to enter via the heme transporter HCP1/PCFT is still hypothetically considered to be exported via the feline leukemia virus subgroup C receptor (FLVCR), a cell surface protein capable of actively exporting heme. Nonetheless, no studies have yet determined the FLVCR function and localization in the enterocyte (Latunde-Dada, Simpson, & McKie, 2006; West & Oates, 2008).

With regard to nonheme iron more is known. After the uptake by the enterocyte it will either constitute, with the iron released from heme, the (i) intracellular labile iron pool (having a molecular nature that is not known but probably consists of low molecular weight chelates or chaperone proteins that bind to and transport iron) (Dunn, Suryo Rahmanto, & Richardson, 2007), or (ii) it can be stored in FT, a cytosolic iron-storage protein (De Domenico et al., 2008). Iron stored as FT will be lost with the senescent enterocytes and the iron from the enterocyte labile iron pool will be, if needed and possible, exported to the plasma through the basolateral membrane transporter ferropotin (FPN) where it will bind to plasma transferrin (TF) (De Domenico et al., 2008; R. E. Fleming & Ponka, 2012; Fuqua et al., 2012; Munoz, Garcia-Erce, & Remacha, 2011).

Ferropotin is the only iron exporter in the duodenal mucosa, macrophages and hepatocytes (De Domenico et al., 2008; Dunn et al., 2007; Zhang & Enns, 2009a). It is likely that FPN exports iron in the ferrous form; however, TF only binds ferric iron (Fuqua et al., 2012; Munoz et al., 2011). In the basolateral membrane, iron export through the FPN is dependent of hephaestin (HP), a ferroxidase that converts ferrous iron to ferric iron and allows it to bind to TF (Zhang & Enns, 2009a). While other cells rely on the circulating ferroxidase ceruloplasmin (CP) for iron oxidation, enterocyte FPN relies on the membrane-bounded paralog HP. Independently of the cell, the feroxidase activity is indispensable for the FPN, since without feroxidase activity FPN is internalized and degraded, preventing iron from being exported and leading to its accumulation in the cell, stored by the FT (De Domenico et al., 2008; Fuqua et al., 2012; Miret et al., 2003).

As previously mentioned, nearly all of the iron exported from the enterocyte is bound to TF, which can bind two molecules of ferric iron. This mechanism of iron chelation dampens the toxicity of the free iron and maintains iron in a soluble form. Under normal circumstances 20 to 30% of the circulating TF binds iron, which is ensured by the high binding affinity of TF to iron and the high concentration of apo-transferrin (apo-TF, iron free form of TF). TF is also responsible for delivering iron to the cells that express TF receptors (Nancy C. Andrews & Schmidt, 2007; De Domenico et al., 2008).

Several mechanisms have been identified to be involved in the cellular TF-dependent iron uptake. The ubiquitously expressed transferrin receptor 1 (TFR1) is the major vehicle for cellular iron uptake and is also the most well studied mechanism of internalization of the diferric-TF complex. Other cellular iron importers have been identified, including the transferrin receptor 2 (TFR2 – expressed in hepathocytes, erythroid cells and duodenal crypt cells) and cubilin (expressed in epithelial cells of the kidney)(Hentze et al., 2004; Hentze, Muckenthaler, Galy, & Camaschella, 2010; Sheftel, Mason, & Ponka, 2012). TFR1 is presented in the cell surface to the plasma as a dimer and binds two diferric-TF molecules. The complex formed by the differic-TF and TFR1 localizes in clathrin-coated pits that are endocytosed. A proton-pump acidifies the early endosome containing the diferric-TF/TFR1 complex, promoting conformational changes in both and releasing iron. Thereafter, the ferric iron released from the complex is reduced to ferrous iron by the ferriredutase STEAP3 and transported to the cytosol by DMT1 (Gkouvatsos, Papanikolaou, & Pantopoulos, 2012; Hentze et al., 2004; Sheftel et al., 2012; Zhang & Enns, 2009a). After the release of the ferric iron, the complex apo-TF/TFR1 returns to the cell surface, the apo-TF is released, and the cycle restarts (N. C. Andrews, 2008; Gkouvatsos et al., 2012; Mayle, Le, & Kamei, 2012).

Once iron enters the cell, the amount of iron that is not needed for immediate use is sequestered from the iron labile iron pool by FT (Hentze et al., 2004; Sheftel et al., 2012). The labile iron pool represents approximately 3 to 5 % of the cellular iron stores and is composed of iron associated to low molecular weight chelates (Gkouvatsos et al., 2012). FT is an heteropolymer of 24 subunits H (heavy or heart) and L (light or liver) types that can encapsulate up to 4500 iron atoms, solubilizing and maintaining them in a less reactive form. Iron stored in FT is readily available for cellular utilization. Thus, in addition to the storage function, FT also has enzymatic properties (oxidization of ferrous iron to ferric iron) (N. C. Andrews, 2008; Nancy C. Andrews & Schmidt, 2007; De Domenico et al., 2008; Hentze et al., 2004).

Intracellular iron can be directed to several sites (for example, to the nucleus, FT or the labile iron pool), but much of it is directed to the mitochondria where the synthesis of heme and iron-sulfur (Fe-S) clusters takes place. Despite of the great biological significance of this process, the mechanism responsible for the traffic of iron to the mitochondria remains unclear (Hentze et al., 2004; Napier, Ponka, & Richardson, 2005). Nonetheless, it is accepted that mitochondrial iron export requires Fe-S cluster biosynthesis. Fe-S clusters and heme are exported from the mitochondria by specific transporters; for Fe-S clusters these are postulated to be the ATP-binding cassette, subfamily B, member 7 (ABCB7) (Dunn et al., 2007; Napier et al., 2005). After being exported from the mitochondria, heme will be used for insertion in several proteins such as hemoglobin and cytochromes, although the mechanism for heme release from the mitochondria remains unclear (Dunn et al., 2007; Hentze et al., 2010; Munoz et al., 2011).

As previously mentioned, in normal conditions, 1 to 2 mg of iron are absorbed by enterocytes each day to compensate the losses, but these amounts only represent a small portion of the body iron daily needs. A total of 3000 to 5000 mg of iron can be present in a healthy adult. The erythron incorporates more than two thirds of the total body iron as hemoglobin; approximately 1800 mg of iron are incorporated in circulating erythrocytes and near 300 mg are present in the bone marrow and in erythroid precursors. Hemoglobin synthesis is responsible for nearly 80% of the iron demands in humans (20 to 25 mg/day) (Hentze et al., 2004; Zhang & Enns, 2009b). The amount of iron that is present in the erythron normally exceeds the iron that is present in iron-stores, which are mainly the hepatocytes (approximately 1000 mg) and the reticuloendothelial macrophages (estimated in nearly 600 mg). A small amount is also present in other cells and tissues, such as muscle cells (300 mg incorporated into myoglobin) and circulating bounded to TF (3 mg) (Nancy C. Andrews, 1999; T. Ganz, 2008; Hentze et al., 2004). The daily demands of iron are supported mainly by the macrophages in the liver and spleen, which phagocytize the senescent erythrocytes and recycle the iron. The iron in the hemoglobin is released, as previously mentioned, by the HO, and exported into circulation through FPN (relying on CP as a ferroxidase) or directly by the FLVCR (Donovan & Andrews, 2004; Munoz et al., 2011; Zhang & Enns, 2009a, 2009b). The circulating heme can be transported to the hepatocytes by the hemopexin and, subsequently, degraded for iron release (Zhang & Enns, 2009a).
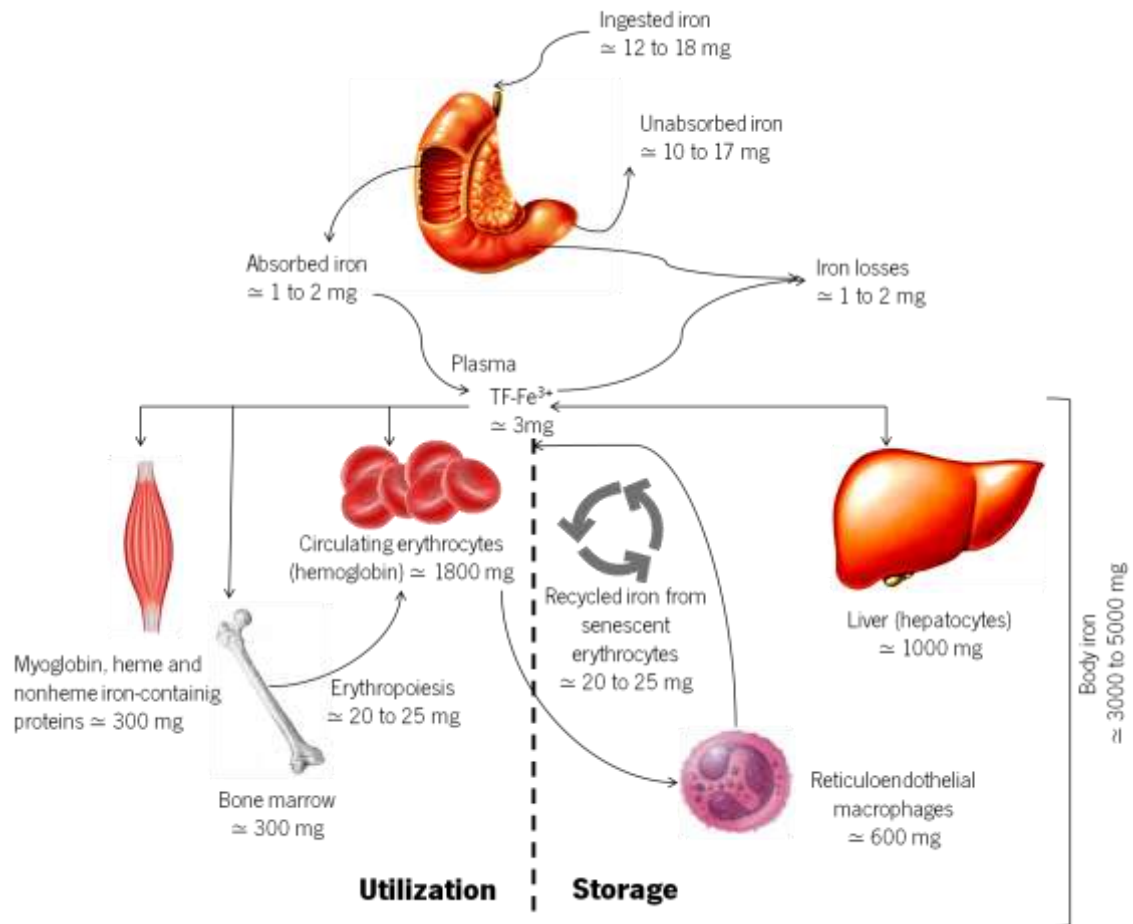
Figure 2 – Adult iron distribution and daily whole body iron metabolism.

Since there is no known mechanism of iron excretion, a highly regulated mechanism of absorption and release from the stores is needed to maintain iron homeostasis at the cellular and systemic levels (Nancy C. Andrews, 1999; T. Ganz, 2008; Hentze et al., 2004). Still, the molecular basis of the complex homeostatic network has only recently been uncovered (T. Ganz, 2008; Simpson & McKie, 2009).

At a cellular level, iron homeostasis is maintained through transcriptional, translational and post-translational mechanisms. The messenger ribonucleic acid (RNA) of several iron related proteins has untranslated regions denoted as iron-responsive elements (IRE). The IREs are found in the 5' untranslated region of the messenger RNA that encodes for FPN, TF chains, mitochondrial aconitase and erythroid 5-aminolevulinic acid synthase (enzyme of heme biosynthesis); whereas, the messenger RNA that encodes for TFR1 and DMT1 have IRE at the 3' untranslated region. The cytosolic proteins iron-regulatory proteins (IRP) recognize and bind to IRE, exerting regulatory effects. The binding of IRP to the IRE in the 5' untranslated regions precludes translation; while the formation of the IRP/IRE complex in 3' untranslated regions

stabilizes the messenger RNA and prevents its degradation (De Domenico et al., 2008; Hentze et al., 2004). Two IRPs have been identified, IRP1 and IRP2, with share sequence homology but have different properties (N. C. Andrews, 2008). In iron-depleted cells the IRP binds to IRE, a process that does not happen in iron-replete cells (T. A. Rouault, 2006). This is possible due to the iron sensing properties of the IRPs. In the presence of high cellular iron levels, a Fe-S cluster will assemble in the IRP1 converting it to an aconitase, inhibiting the binding of IRP1 to IRE. On the contrary, in iron depleted cells the absence of the Fe-S cluster allows IRP1 to be in its RNA-binding form. The IRP2 does not contain the Fe-S cluster and seems to have lost the aconitase activity along the evolutionary process. While in iron-depleted cells the IRP2 accumulates, in iron-replete cells it is targeted for degradation (N. C. Andrews, 2008; De Domenico et al., 2008; Hentze et al., 2004; T. A. Rouault, 2006). Iron cytosolic concentration is rapidly adjusted by the IRP/IRE regulatory system and the function of several iron-dependent components and processes is therefore optimized (T. A. Rouault, 2006).

At a systemic level several signals such as iron absorption ("mucosal block"), oxygen tension (hypoxia regulator), iron levels (stores regulator) and systemic iron needs (erythroid regulator) regulate iron absorption, storage and transfer (R. E. Fleming & Ponka, 2012; Hentze et al., 2004; Simpson & McKie, 2009). Circulating iron status is maintained at balanced levels by two main mechanisms: (i) absorption regulation, and (ii) regulation of release by the macrophages and hepatocytes (T. Ganz, 2008). In 1960, the hypothesis that a humoral substance was responsible for match iron absorption to the iron demands was put forward by Beutler *et al.* (Beutler & Buttenwieser, 1960). However, only in 2001 Nicolas et al. (Nicolas et al., 2001) demonstrated that hepcidin, the peptide sequenced in 1998 by Ganz and collaborators (Park, Valore, Waring, & Ganz, 2001), had an iron regulator function that made it a leading candidate for the long-sought iron-regulatory hormone (Robert E. Fleming & Sly, 2001). The small size of its gene, the lack of shared sequence motifs with other iron related genes and the rarity of mutations prevented the discovery of hepcidin by genetic methods, but several serendipitous events helped to bring hepcidin and its function to the knowledge of the scientific community (Tomas Ganz, 2011). Hepcidin, a 25 amino acid peptide hormone is the principal systemic regulator of intestinal iron absorption and iron efflux from macrophages. This hormone is primarily produced by the hepatocytes in the liver and in smaller amounts by other cell types upon cleavage of a pro-peptide hormone with 84 amino acids by the convertase furin. The biological action of hepcidin is exerted by its binding to FPN, which leads to FPN phosphorylation,

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internalization and ubiquitin-mediated lysossomal degradation. Since FPN is the only exporter of iron from enterocytes, macrophages and hepatocytes, its degradation results in a decrease of the delivery of iron from the enterocytes and the reticulo-endothelial system to the plasma (N. C. Andrews, 2008; Fuqua et al., 2012; T. Ganz & Nemeth, 2006; Gkouvatsos et al., 2012). A similar mechanism has been recently proposed to occur at the barriers of the brain, in this case regulating iron delivery into the central nervous system (Marques et al., 2009).

Hepcidin is negatively regulated by iron (circulating and stores), erythropoietic needs, inflammation and hypoxia. (Tomas Ganz, 2011; Gkouvatsos et al., 2012). Specific conditions, such as inflammation, are potent suppressors of iron absorption by enterocytes and release by macrophages. There is evidence that this effect is mediated by hepcidin since inflammatory cytokines, with special emphasis on IL-6, activate hepcidin expression. This mechanism is the major contributor to the anemia of chronic disease (ACD – also called anemia of chronic inflammation) (T. Ganz & Nemeth, 2006; Hentze et al., 2010). Iron absorption is regulated in several different manners, being the iron from diet the first regulator. After the ingestion of a significant amount of dietary iron, the enterocyte becomes refractory to iron absorption. This phenomena, so-termed "mucosal block", probably results from an accumulation of intracellular iron that will change the levels of DMT1 and DCYTB, which will prevent further iron uptake (Nancy C. Andrews, 1999; De Domenico et al., 2008). Iron absorption is also regulated in cases of normoxia and hypoxia by the hypoxia inducible factor (HIF), a nuclear transcription factor first described as the major oxygen regulated transcription factor through the control of erythropoietin expression and, therefore, of oxygen supply to red cell production (Simpson & McKie, 2009). HIF is also described to regulate *Hepcidin* expression. Recently, compelling data has indicated that *DCYTB, DMT1* and possibly *FPN* genes, which are highly up regulated in hypoxia and iron deficiency, are also HIF-2α dependent for expression (Mastrogiannaki et al., 2009; Shah, Matsubara, Ito, Yim, & Gonzalez, 2009). (Nancy C. Andrews, 1999; Han, 2011).

In summary, iron homeostasis is a very highly regulated process for which several pathways and molecular players participate.

1.1.2. Assessment of iron status

Bone marrow

Bone marrow grading is the gold standard for iron deficiency assessment and provides the definitive diagnosis. The major limitations of this method are the high invasiveness, cost (expensive) and pain incurred by the assessment procedure. Therefore, bone marrow aspiration is rarely performed and alternative sensitive and less invasive tests are used (Fairweather-Tait, Wawer, Gillings, Jennings, & Myint, 2013; Gale, Torrance, & Bothwell, 1963; G. H. Guyatt et al., 1992; Raiten et al., 2011; Rimon, Levy, Sapir, & et al., 2002).

Hemoglobin

The assessment of the prevalence of ID in developed countries is commonly obtained from representative samples with specific indicators of iron status, such as serum FT, TF saturation and free erythrocyte protoporphyrin; whereas, estimates from developing countries are commonly based in hemoglobin measurements (Zimmermann & Hurrell, 2007). Iron homeostasis is a continuum that ranges from iron overload to iron depletion being ID and IDA intermediate states (Figure 3), (WHO, 2001).
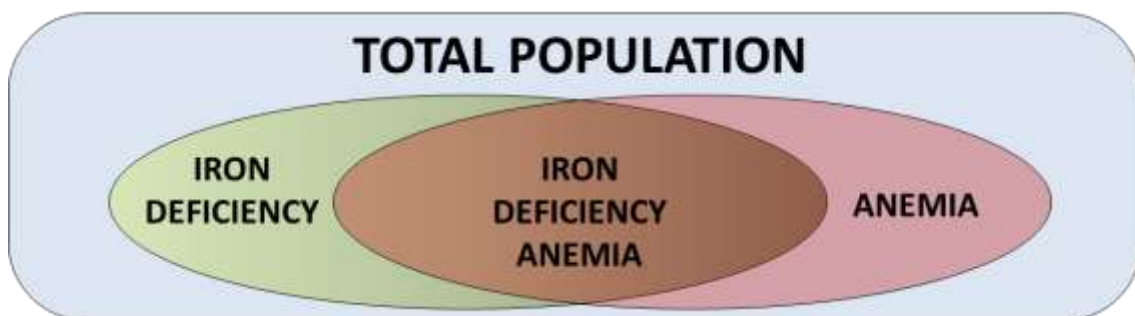
Figure 3 - Conceptual diagram of the relationship between iron deficiency and/or anemia in a hypothetical population. Adapted from WHO (2001).

Despite of the low sensitivity and specificity of hemoglobin as an indicator of ID, its levels are commonly used in surveys of anemia (of all causes) (Lynch, 2010). Nevertheless, anemia (of all causes) surveys for assessment of ID are inadequate since they do not properly correlate with each other, which may justify the limited success of public health programs based on hemoglobin levels (Cook, Flowers, & Skikne, 2003; Mei et al., 2005). Underlying this may be that anemia has multiple precipitating factors in addition to iron deficiency, such as genetic disorders,

infectious diseases and other nutritional deficiencies (McLean et al., 2009; Pang & Schrier, 2012; Patel, 2008), which may differ between places. Therefore, anemia, IDA and ID are commonly, although not correctly, used as synonymous. Despite this, the overall prevalence of ID is estimated to be equal to the prevalence of anemia (of all causes), since it is assumed that IDA represents 50% of all anemias and the prevalence of ID alone is considered equal to that of IDA (Susan F Clark, 2009; De Benoist et al., 2008; Lynch, 2010; McLean et al., 2009). As previously mentioned, the assessment of iron status in developing countries is mainly preformed using hemoglobin measurements and anemia as a proxy; accordingly, it is thus stated that iron deficiency is the main cause of anemia in underprivileged environments (WHO, 2001).

Inadequate supply of iron to the erythroid marrow over time will lead to an inadequate support for optimal erythropoiesis in the developing red cell mass, which will reduce hemoglobin concentrations to below-optimal levels and, consequently, cause anemia (Bainton & Finch, 1964). Specifically, the WHO classifies hemoglobin values under 12 and 13 g/dL for adult non-pregnant women and adult males, respectively, as defining anemia. These values were defined as under two standard deviations (-2SD) of the distribution mean for hemoglobin in a normal population of the same gender and age living at the sea level. As such, anemia definition is largely a statistical definition rather than a physiological definition (WHO, 2001). It also important to note that a hemoglobin value overlap can occur between persons with iron deficiency and normal non-anemic individuals. Taking into account all the limitations of hemoglobin levels (or anemia cutoff points) for ID assessment it is clear that hemoglobin should not be used as a stand-alone indicator (Gibson, 2005).

Hematocrit and red cell indices

Hematocrit (or packaged cell volume) and red cells indices are often used for differential diagnosis of anemia, which include also iron deficiency anemia. Hematocrit, along with hemoglobin, is used for anemia diagnosis. In cases of early ID a nearly normal hematocrit value can be observed; whereas, in cases of mild to severe ID, both hemoglobin and hematocrit are below the threshold of normality. Several limitations are described for hematocrit use in ID determination. Briefly, hematocrit falling only occurs in a later stage of ID, thus resulting in poor sensitivity. Furthermore, the same factors that affect hemoglobin also affect hematocrit, so lower values of hematocrit can be observed due to causes other than ID, resulting in a poor specificity, and several internal and external factors can affect its value resulting in poor precision. Cutoff

points for hematocrit values are also defined; however, and similarly to hemoglobin, they do not specifically identify iron deficient individuals (Gibson, 2005).

Red cell indices are calculated with the values measured for hemoglobin, hematocrit and red cells blood Count (RBC) and include mean corpuscular volume (MCV), mean cell hemoglobin (MCH), mean cell hemoglobin concentration (MCHC) and red cell distribution width (RDW). MCV can be manually calculated as the hematocrit (volume fraction) divided by the RBC (10^{12}/L) and therefore is a measure of the average size of red blood cells (expressed in femtoliters – fL). In iron deficiency, red cells can be small (microcytosis). However, other nutritional disorders can also affect MCV such as vitamin B_{12} and folic acid deficiencies (both causing abnormal large cells - macrocytosis) and vitamin B_6 (also causing small red blood cells). The ratio of hemoglobin (g/L) to RBC defines the MCH and is expressed in picograms (pg). Changes in MCH occur in the same direction as MCV either in iron deficiency or in vitamin B_{12} and folic acid deficiencies. MCHC can be obtained dividing the hemoglobin by hematocrit and is a measure of the mean hemoglobin amount in each red blood cell. This is the red cell index less affected by age; nonetheless, it is also the least useful for iron deficiency identification because it is the last value to fall. RDW is a measure of the variation of size in red cells and is usually increased in iron deficiency. Again, alterations in RDW can occur in several pathologies and nutritional deficiencies, so RDW is also not a specific indicator of iron deficiency (Gibson, 2005).

Serum iron, total iron binding capacity and transferrin saturation

In the fasting state serum iron levels reflect the migration of iron to bone marrow from the reticulo-endothelial system indicating the iron supply adequacy for erythropoiesis (Fairweather-Tait et al., 2013; Gibson, 2005). Reliability of serum iron is questionable since it is affected by diurnal rhythms, ingestion of iron and inflammatory states (Fairweather-Tait et al., 2013). A useful indicator used for iron deficiency diagnosis is the total iron binding capacity (TIBC), which reflects the amount of serum iron binding sites that are not occupied. TIBC is increased early in iron deficiency (even before iron-deficient supply for erythropoiesis) as a result of the increase in TF production needed for higher iron transport and uptake. TF saturation is calculated from serum iron and TIBC [as follows: serum iron (µg/dL) / TIBC (µg/dL)] and, similarly to serum iron, is a measure of iron supply to erythropoietic tissues (Fairweather-Tait et al., 2013; Gibson, 2005).

Serum ferritin

Tissue ferritin is an intracellular protein involved in iron storage. Serum ferritin is slightly different than tissue ferritin and contains little or no iron (Worwood, 1990). Serum FT function in the serum is unknown, however it seems to be released from the reticulo-endothelial system (Gibson, 2005). Serum FT is the only iron status indicator that can be used to identify normal, deficient and excessive iron status because its concentration is closely related to body iron storage (1 µg/L of serum FT correspond to 8-10 mg of iron stored). Despite of this close correlation, when iron stores are depleted serum FT remain low but no longer directly reflects the severity of tissue iron deficiency (Gibson, 2005; Raiten et al., 2011; Zhu, Kaneshiro, & Kaunitz, 2010). This is also considered to be the single best laboratory test for the diagnosis of iron deficiency since its predictive value is of 0.95 (G. H. Guyatt et al., 1992; Zhu et al., 2010). Due to its high predictive value, simple cutoff points have been established for iron deficiency diagnosis: values lower than 15 µg/L define iron deficiency, while values higher than 100 µg/L rule out iron deficiency and the intermediate values need further investigation (G. H. Guyatt et al., 1992). However, serum FT is influenced by age and is also an acute phase protein and, thus, in case of inflammation serum FT should not be used as an iron status marker (Fairweather-Tait et al., 2013; Raiten et al., 2011; Zhu et al., 2010). For older individuals values lower than 45 µg/L should be used for iron deficiency diagnosis (Gordon H Guyatt et al., 1990). A cutoff point of 30 µg/L for the general population is usually used in the majority of the diagnostic studies of iron deficiency (Johnson-Wimbley & Graham, 2011).

Soluble serum transferrin receptors

As previously described, when an insufficiency of intracellular iron occurs, the expression of TFR-1 on the cell surface is up-regulated and allows for a higher iron uptake. In human serum the concentration of the soluble serum TFR (sTFR), a soluble form of the transmembrane TFR, is proportional to the expression of TFR (Gibson, 2005), rendering it possible to use sTFR as marker of iron deficiency and erythropoietic activity (Fairweather-Tait et al., 2013). Bone marrow erythroid precursors are the main source (75 to 80%) of sTFR and, unlike serum ferritin, sTFR levels are not affected by age or inflammatory conditions (Susan F Clark, 2009; R. Stewart & Hirani, 2012; Zhu et al., 2010). Despite of the usefulness and reliability of sTFR, the assay is not standardized, is expensive and not widely available, which prevents its clinical application (Choi et al., 2005; Zhu et al., 2010). Furthermore, folate and/or vitamin B_{12} affect erythropoiesis and

consequently sTFR concentrations, confounding the interpretation of the assay for iron status assessment (Gibson, 2005).

Ratios and algorithms

The ratio of sTFR to the logarithmic value of ferritin (sTFR – Log(FT) ratio) is also considered a good indicator for the diagnosis of iron deficiency, especially in cases where iron deficiency occurs in combination with other morbidities that elicit an acute phase reaction (for example, elevation of C-reactive protein) (Susan F Clark, 2009; Punnonen, Irjala, & Rajamaki, 1997).

The Cook algorithm (Cook et al., 2003) for body iron calculation (body iron (mg/kg) = -[log(sTFR*1000/serum FT) – 2.8229/0.1207]) is an index for iron status alterations. It is relatively new and can be used as an epidemiological measure for monitoring populations where inflammation has been excluded or is rare, and it can also be used as a measure of effectiveness in intervention studies (Fairweather-Tait et al., 2013).

Use of multiple indicators of iron status

Indicators of iron deficiency have limitations and the use of a single measurement can lead to an erroneous classification of the iron status due to the overlap of normal and abnormal values of the measurement depending on the confounding factors (e.g.: age, inflammatory states or nutritional deficiencies). Thus, the use of multiple indices can be a more reliable alternative to identify abnormalities of iron metabolism. Usually, a combination of three indicators is used and abnormal values for at least two rules in iron deficiency (Gibson, 2005). However, due to the high predictive value, low values of ferritin have been used as a single indicator for iron deficiency in the absence of inflammation (G. H. Guyatt et al., 1992; Raiten et al., 2011; Rimon et al., 2005). Furthermore, serum ferritin concentration is the most powerful test for iron deficiency (Goddard, McIntyre, & Scott, 2000). Table 1 provides a summary of the principal indices used for iron assessment, the normal values and the cutoff points for iron deficiency.

Table 1 - Cut off points for ID diagnosis. Adapted from Susan F Clark, 2009; Gibson, 2005; G. H. Guyatt et al., 1992; Gordon H Guyatt et al., 1990; Herbert, 1987; Johnson-Wimbley & Graham, 2011; Punnonen et al., 1997; Rimon et al., 2002; Zhu et al., 2010; Zimmermann & Hurrell, 2007

Measurements	Normal	I. D.
MCV(fL)	80-100	<80
MCHC (g/L)	320-360	<320
RDW (%)	≤14	>14
Serum iron (µg/dL)	115±50	<71
Serum ferritin (µg/L)	100±60	<30/<45 [a]
Transferrin saturation (%)	35±15	<20
TIBC (µg/dL)	330±30	≥360
sTFR (mg/L)	≤1.76	>1.76
sTFR – Log(FT) ratio	≤1.5	>1.5

[a] Aged individuals

1.2. Iron deficiency

1.2.1. From basics to clinics

In 1937, McCance and Widdowson observed that there is no mechanism for iron excretion, implying that homeostasis is maintained by the regulation of absorption (Hallberg, 2001; Miret et al., 2003). It is estimated that daily 1 to 2 mg of dietary iron is absorbed, which is enough to compensate the continuum losses that results from cell shedding and fluids loses. As previously mentioned, iron homeostasis is narrowly regulated at the absorptive level; although, even in iron overload, 0.5 mg of iron are absorbed and in iron deficiency, despite of the increase in absorption, only 2 to 4 mg are absorbed (Miret et al., 2003). Inadequate iron intake and absorption are the main causes of ID, particularly when they are insufficient to overcome physiologic needs and/or pathologic conditions (i.e.: blood losses) (see Table 2 for details). Each 1 ml of blood contains approximately 0.5 mg of iron, therefore the occurrence of significant blood losses (due to hemorrhagic events or excessive menorrhea) markedly increases the risk of iron deficiency (Zimmermann & Hurrell, 2007) and the small amounts of iron absorbed make it time consuming to revert.

Table 2 - Physiologic and pathologic conditions that can cause ID or IDA. Adapted from Nancy C. Andrews, 1999; Susan F. Clark, 2008; Susan F Clark, 2009; Munoz, Garcia-Erce, & Remacha, 2011b

Physiologic conditions	Pathological conditions
Increased iron/dietary demands	Chronic inflammatory states
Pregnancy	Chronic renal disease
Lactation	Congestive heart failure
Infants	Obesity
Children and adolescents	Medications
Menstrual losses	Proton-pump inhibitors
	H_2 antagonists
	Antacids
	Aspirin
	NSAIDs
	Gastrointestinal conditions
	Ulcer (gastric/duodenal/peptic)
	Gastritis (Erosive/*H. pylori*)
	Chronic atrophic gastritis
	Esophagititis
	Celiac/Chrohn's disease
	Malabsorptive states
	Gastrectomy
	Colonic adenoma
	Carcinoma
	Blood losses
	Excessive menstrual losses
	Blood donation
	Excessive surgical blood losses
	Inflammatory bowel disease
	Other
	Hepatic disease
	Restless leg syndrome
	Athletic induced ID
	Intestinal helminthes
	Erythropoiesis-stimulating agents
	Malaria
	Human Immunodeficiency Virus

Previous studies on ID mainly focused on infants, children and women of childbearing age. Only a handful of studies on the elderly have been conducted (Hsu et al., 2013). In aging, although unlikely that physiologic conditions will be responsible for the development of ID late in life, several of the iron-related/caused pathological conditions are highly prevalent in older individuals (Susan F. Clark, 2008). Hypochlorhydria can be a substantial cause of iron deficiency given the low production of acid in the stomach (needed to keep iron soluble); it is highly

prevalent in older individuals due to several causes, such as atrophic gastritis, medication (e.g.: proton-pump inhibitors, antacids and H_2 antagonists) and *Helicobacter pylori* (Annibale et al., 1999; Susan F. Clark, 2008; Zimmermann & Hurrell, 2007). The mismatch between iron absorption and iron demands is also observed in gastrointestinal conditions, including ulcers, inflammatory bowel diseases, both by bleeding or absorption impairment (Annibale et al., 1999; Gasche, Lomer, Cavill, & Weiss, 2004).

In hepatic conditions, iron deficiency has also been observed since the liver is the main storage place of iron as FT and the site of TF synthesis. Ultimately, in liver diseases iron may become trapped and therefore not released for transportation to other tissues (Susan F. Clark, 2008; Intragumtornchai, Rojnukkarin, Swasdikul, & Israsena, 1998). Also, in cirrhotic patients the absorption of some micronutrients is compromised, contributing for nutritional deficiencies among which iron deficiency is common (Susan F. Clark, 2008; Peng et al., 2007).

The inadequate delivery of iron for incorporation in the erythroid precursors despite of an adequate storage of iron in the bone marrow is termed functional iron deficiency. This type of "iron deficiency" is present in some cases such as malignancy, anemia of chronic disease (ACD – also called anemia of chronic inflammation) or chronic kidney disease (Thomas et al., 2013). ACD is observed both in infectious and noninfectious diseases. The linkage between ACD and iron deficiency is mediated by hepcidin and inflammatory cytokines (such as interleukin-6). In infection and inflammation the production of hepcidin is upregulated resulting in hypoferremia (Nancy C. Andrews, 1999; N. C. Andrews, 2008; Brugnara, 2003; T. Ganz & Nemeth, 2006). The risk to develop chronic kidney disease is higher in older individuals, and subjects with chronic kidney disease are at a higher risk of developing iron deficiency, especially if under hemodialysis (due to increased iron loses) or treatment with erythropoiesis stimulating agent (due to the increased iron demands) (Susan F. Clark, 2008; Wittwer, 2013). Recently, it was also demonstrated that patients with heart failure may be more susceptible to iron deficiency due to depletion of iron stores, defective iron absorption and/or reduced availability of iron recycled in the reticuloendothelial system (Nanas et al., 2006; Opasich et al., 2005).

Among others, all of the above-mentioned factors may be highly prevalent in older individuals and, individually or by their interaction can alter iron homeostasis and mediate the development of iron deficiency.

1.2.2. Epidemiology of iron deficiency

Iron deficiency is the major cause of anemia. The WHO Department of Nutrition for Health and Development, based on the Vitamin and Mineral Nutrition Information System (VMNIS), has estimated anemia prevalence at global, regional and national levels (De Benoist et al., 2008; Mathers et al., 2008). The most recent data available regarding IDA prevalence was published in the WHO report *The Global Burden of Disease: 2004 Update (2008)* (Mathers et al., 2008)), in which it is estimated that IDA corresponds to 60% of all anemias in non-malaria areas and 50% in areas were malaria is prevalent (given the high hemolysis present) (Mathers et al., 2008). In fact, IDA, at any moment, is the most prevalent health condition. A prevalence equal or higher than 5% is considered a public health problem (WHO, 2001). In 2004, it was estimated that 1 159 millions (in a population of 6 437 millions) of individuals across the world have IDA, which accounts for a prevalence rate of 18% (Table 3). Despite of Africa and South East Asia contributing to more than a half (56.6%) of the cases, IDA is highly prevalent in every WHO region. Europe presents the second lowest prevalence of IDA. These estimates are made based on nonspecific markers, such as hemoglobin and predictive equations (McLean et al., 2009) and, therefore, data should be interpreted with caution.

Table 3 - Prevalence of IDA in WHO regions, 2004

	Cases of IDA (millions)	Population (millions)	Prevalence (%)
World	1 159.3	6 437	18.0
Africa	193.8	738	26.3
South East Asia	462.4	1 672	27.7
The Americas	66.4	874	7.6
Eastern Mediterranean	88.5	520	17.0
Europe	77.7	883	8.8
Western Pacific	269.0	1 738	15.5

The main body of literature regarding irons status in European countries dates back to the 80s and early 90s, with authors basing their estimates in hemoglobin determination and only a few reporting data from southern European countries. Also, no data are available for older individuals probably due to the high number of confounding factors present in this population group (Hercberg, Preziosi, & Galan, 2001). In the United States of America, the Centers for Diseases Control and Prevention, using specific markers of iron status (serum FT, TF saturation and free erythrocyte protoporphyrin), in the population sample examined in the National Health

and Nutrition Examination Survey (NHANES) 1988-1994 and 1999-2000, reported a prevalence of iron deficiency in older individuals that ranged from 3% to 9% and a significant increase in iron deficiency prevalence in females between the ages of 50 to 70 years (Control & Prevention, 2002).

Epidemiological data regarding death and disability adjusted life years (DALY) was recently updated in the Global Health Estimates for the years 2000–2012 ("WHO | Estimates for 2000–2012," 2014). In Europe, IDA as a cause of death only overcomes the barrier of thousands in aged individuals strata (60 or years or older), with a higher expression in females, which is expected since there are more females in that age group (Table 4). Interestingly, despite of being in older individuals that ID is more life threatening, this is the population group in which the effects of ID are less studied.

Table 4 - Deaths (population in thousands) due to IDA by age and sex, in WHO regions. Global health estimates 2012 ("WHO | Estimates for 2000–2012," 2014).

	Males			Females			Total
	0-14	15-59	60+	0-14	15-59	60+	
World	8.711 (963.536)	14.686 (2.230.904)	16.692 (371.994)	9.062 (899.757)	17.030 (2.167.894)	23.980 (441.372)	90.160 (7.075.457)
Africa	6.863 (191.908)	10.576 (233.995)	5.092 (20.234)	7.192 (187.547)	12.137 (234.858)	7.070 (23.986)	48.930 (892.528)
South East Asia	330 (276.787)	564 (588.968)	847 (71.593)	391 (253.799)	1.045 (561.881)	2.034 (80.333)	5.212 (1.833.361)
The Americas	890 (119.163)	1.512 (293.118)	4.799 (58.664)	769 (114.236)	1.420 (299.602)	6.157 (71.996)	15.546 (956.779)
Eastern Mediterranean	275 (104.502)	397 (191.473)	316 (19.011)	305 (98.971)	468 (178.161)	415 (20.252)	2.175 (612.370)
Europe	54 (81.140)	152 (279.788)	1.773 (76.324)	37 (77.066)	158 (283.617)	3.504 (106.549)	5.678 (904.484)
Western Pacific	298 (186.954)	1.448 (633.244)	3.781 (123.978)	366 (165.275)	1.778 (599.502)	4.704 (135.797)	12.374 (1.844.750)

Since iron deficiency is more prevalent in Africa and South East Asia the burden of iron deficiency (Table 5) is higher in these regions. Cost effectiveness of public health strategies and the burden of iron deficiency are measured in cost per DALY saved (Horton, 2006; Longfield, Smith, Gray, Ngamkitpaiboon, & Vielot, 2013), therefore low income countries will be the ones in which fortification will result in higher economic gains. Interventions to improve iron status have also been proposed to improve human capital since work capacity is reduced in iron deficiency (Haas & Brownlie, 2001).

Table 5 - Burden of disease in DALYs (population in thousands) due to IDA by age and sex, in WHO regions. Global health estimates 2012 ("WHO | Estimates for 2000–2012," 2014).

	Males			Females			Total
	0-14	15-59	60+	0-14	15-59	60+	
World	14.596.920 (963.536)	5.215.420 (2.230.904)	1.163.788 (371.994)	12.790.183 (899.757)	12.033.058 (2.167.894)	1.827.788 (441.372)	47.627.157 (7.075.457)
Africa	4.286.630 (191.908)	1.752.296 (233.995)	238.619 (20.234)	3.758.726 (187.547)	2.640.851 (234.858)	301.709 (23.986)	12.978.831 (892.528)
South East Asia	5.358.228 (276.787)	2.104.376 (588.968)	451.869 (71.593)	4.821.630 (253.799)	5.781.635 (561.881)	751.513 (80.333)	19.269.251 (1.833.361)
The Americas	1.168.139 (119.163)	418.009 (293.118)	169.581 (58.664)	743.312 (114.236)	518.521 (299.602)	143.455 (71.996)	3.161.017 (956.779)
Eastern Mediterranean	1.753.883 (104.502)	542.680 (191.473)	99.294 (19.011)	1.478.751 (98.971)	1.205.345 (178.161)	123.502 (20.252)	5.203.455 (612.370)
Europe	612.534 (81.140)	212.935 (279.788)	108.048 (76.324)	493.197 (77.066)	698.247 (283.617)	203.093 (106.549)	2.328.054 (904.484)
Western Pacific	1.391.653 (186.954)	177.884 (633.244)	93.408 (123.978)	1.466.129 (165.275)	1.153.815 (599.502)	296.026 (135.797)	1.662.945 (944.176)

1.2.3.   General health

Iron deficiency will lead to iron deficient erythropoiesis, which ultimately will result in iron deficiency anemia. Therefore, the health outcomes of iron deficiency include the ones associated with anemia. Traditionally, iron deficiency has been considered to have clinical consequences only in the presence of anemia (Anker et al., 2009). However, several examples demonstrate beneficial effects of iron treatment even in non-anemic patients; such is the case of iron treatment in non-anemic subjects with restless leg syndrome who presented greatly reduced symptomatology (Satija & Ondo, 2008).

In older individuals, several deleterious health consequences are associated with anemia including physical performance decline, frailty, higher risk of falling, diminished quality of life, cardiovascular disease, cognitive impairment, morbidity, hospitalization and mortality (Beghe, Wilson, & Ershler, 2004; Chaves, 2008; Fairweather-Tait et al., 2013; Hsu et al., 2013; Pang & Schrier, 2012; Patel, 2008; Peters et al., 2008; Price, Mehra, Holmes, & Schrier, 2011; Roy, 2011; M. Thein et al., 2009; Van Puyvelde, Cytryn, Mets, & Beyer, 2009). Evidence clearly and consistently demonstrates the existence of an anemia association with poor health outcomes. However, the inference about causality is restricted due to the inherent limitations of observational studies, which compose the main body of literature on this topic (Chaves, 2008). For example, Hsu *et al.* (Hsu et al., 2013) found that iron deficiency, measured by serum iron, was associated with cardiovascular disease and all causes of mortality; although, several

limitations make the interpretation and causality of this study difficult. Using a more specific, but limited, set of iron biomarkers in a larger cohort, Morkedal *et al.* (Mørkedal, Laugsand, Romundstad, & Vatten, 2011) reported an association of low iron status with an higher risk of death by ischemic heart disease, particularly if low iron status was observed in earlier stages of the follow-up. The authors suggested that low iron status may be a late sign of ischemic heart disease or that unknown prevalent disease at baseline could influence the associations between low iron status and death. In a prospective study in Finland (Marniemi et al., 2005), the relative risk of acute myocardial infarction was diminished in individuals in the highest tertile of baseline serum iron and the ones in the middle tertile had a reduced risk of stroke. The ones in the highest tertile of serum TF presented an increased risk of stroke. Of note, other reports have failed in demonstrating these associations (Jia, Aucott, & McNeill, 2007).

For the observed associations, several factors/indicators/mechanisms can plausibly explain the association of iron deficiency and cardiovascular disease and mortality. Iron deficiency may reflect undiagnosed conditions since iron deficiency is associated with renal failure, cancer and inflammation that *per se* are associated with higher mortality rates. Malnutrition is associated with increased mortality and an iron deficient state could be a surrogate marker for it (Fairweather-Tait et al., 2013; Hsu et al., 2013).

Iron deficiency has also been implied in regulation of body temperature related to secretion and utilization of thyroid hormones (Boccio & Iyengar, 2003). It is also known that iron deficiency impacts on immune function and in resistance to infections; however, the results on this topic are somehow conflicting. Some studies suggest beneficial effects of mild iron deficiency on resistance to infection while others report that, independently from the severity, iron deficiency is always deleterious for immune function (Oppenheimer, 2001; Walter, Olivares, Pizarro, & Muñoz, 1997).

1.2.4.   Physical performance and functional ability

The association of anemia and physical functional ability has been highlighted in several reports in the last years (Denny et al., 2006; Lucca et al., 2008; Onem et al., 2010; Penninx et al., 2003; Penninx et al., 2004; M. Thein et al., 2009). Although hemoglobin is used as a marker for iron status, no conclusions can be drawn from these studies on the impact of iron deficiency in physical functional ability. Still, theoretically, iron deficiency can impair physical functioning of older adults. Also of interest, subjects with iron deficiency present a 50% decrease in muscle

myoglobin content, electron transport capacity and cytochorme oxidase activity and the delivery of oxygen to muscle is limited in anemia (J. L. Beard, 2001; P. Dallman, 1982; P. R. Dallman, 1986; Davies, Maguire, & Brooks, 1982).

The effects of iron deficiency *per se* in the physical performance and functional ability of older individuals have yet to be elucidated. A critical review (Haas & Brownlie, 2001) of the effects of iron deficiency on work capacity hypothesized that severe and moderate iron deficiency anemia has a causal effect on reduced work capacity. Endurance capacity was also compromised in severe and moderate iron deficiency anemias and energetic efficiency was affected in all levels of iron deficiency (severe and moderate iron deficiency anemia and iron deficiency without anemia).

1.2.5.   Brain and cognition

The central nervous system, along with retina and the testis, is independent of the liver regulatory axis of iron homeostasis (Tracey A. Rouault & Cooperman, 2006). The movement of iron from plasma to cerebrospinal fluid seems to be regulated by the blood-brain and the choroid plexus-cerebrospinal fluid barriers (Marques et al., 2009). Similar to the intestinal absorption, iron uptake to the brain is decreased when the iron status is high and increased when it is low, in a process that is highly selective (J. Beard, 2003; Ke & Qian, 2007). Also, the distribution of iron in the brain is not equal in all regions with some (namely: basal ganglia, substantia nigra and deep cerebellar nuclei) particularly rich in iron, some even richer than the liver (J. Beard, 2003; M. H. Youdim, 2008). Iron homeostasis is paramount for normal brain function and brain iron deficiency will disrupt several important processes, possibly altering neurochemistry and conducting to a pathological state. Still, studies on the effects of iron deficiency on brain function only in recent years have received attention from the scientific community (Hare, Ayton, Bush, & Lei, 2013; M. H. Youdim, 2008), with the majority of these with focus in infants and in animal models (J. Beard, 2003; Ke & Qian, 2007; Mesquita et al., 2012).

Within the brain, iron has an important role in metabolism, including in processes such as synthesis, packaging, uptake and degradation of neurotransmitters. Furthermore, it can indirectly affect brain function through peroxide reduction, amino acid metabolism and membrane functioning alteration by fat desaturation (J. L. Beard, 2001). Although the molecular bases are not completely understood (Fretham, Carlson, & Georgieff, 2011; Muñoz & Humeres, 2012), it is known that iron deficiency has time-, duration- and severity-dependent deleterious

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effects on gene profiling, myelination, neurotransmission, learning and memory. The major body of human studies on this topic focuses in the developmental periods, but the effects of iron deficiency in later ages were not carefully examined (J. Beard, 2003). Still, in some cases, the effects of iron deficiency during developmental periods can be observed even in adults and are supported by the irreversible effects observed in animal models of development (J. Beard, 2003; Lozoff, 2007; Lozoff et al., 2006). Furthermore, although development seems to be a critical period, there is evidence that the deleterious effects of iron deficiency on neuronal function, behavior and cognitive function are not restricted to early ages (J. Beard, 2003; Bruner, Joffe, Duggan, Casella, & Brandt, 1996; Muñoz & Humeres, 2012). In older children and in adults, observational evidence suggests a strong link between iron deficiency and cognitive deficit, and the alterations can be reverted by the correction of iron deficiency, suggesting that time of iron deficiency matters (J. Beard, 2003; J. L. Beard et al., 2005; Bruner et al., 1996; Falkingham et al., 2010).

Iron deficiency and its associations with cognition in older individuals remain largely unexplored. In fact, only one report on this age strata was found in the literature. In a cross sectional study, Yavuz *et. al.* (Yavuz et al., 2012) found an association between ID and mini-mental state examination (MMSE); however, the statistical analysis was not controlled for confounding factors such as age and education. Falkingham et al. (Falkingham et al., 2010), in a systematic review and meta-analysis of randomized controlled trials of iron supplementation on cognition, found some evidence that iron supplementation improved attention, concentration and intelligence quotient, but no studies included men, post-menopausal women or the elderly.

1.2.6.   Mood

It is widely accepted that certain micronutrient deficiencies can be associated with depression and/or depressive mood, and that psychological morbidity can affect appetite and nutritional ingestion (Sarris, Schoendorfer, & Kavanagh, 2009). In older individuals, anemia *per se*, even in the absence of iron deficiency, has been associated with depressive symptomatology (Hamer & Molloy, 2009; Onder et al., 2005). Iron deficiency has also been associated with depressive symptoms in premenopausal women (Laura E. Murray-Kolb, 2011). Yet, there is remarkably little research regarding neuropsychological morbidity and iron status especially in older subjects. To our knowledge, the only study measuring this association was conducted by

Stewart and Hirani (R. Stewart & Hirani, 2012), who reported a significantly higher depressive mood in individuals with anemia, low ferritin and high sTFR after controlling for age, sex, social class, multivitamin intake, smoking status and body mass index (BMI).

1.3.   T$_{(h)}$reat$_{(en)}$ the iron deficiency

Iron deficiency is treatable and preventable (Miller, 2013). While treating iron deficiency the focus should be targeting the underlying cause of it, which is often difficult to determine, and the main goal should be the repletion of iron stores (Goddard et al., 2000; Mahan, Escott-Stump, Raymond, & Krause, 2012). In order to replenish iron stores of adults, oral administration of iron is the first line of treatment and, depending on the severity of the case, 50 to 200 mg of inorganic iron, in the ferrous form, are daily prescribed and should be maintained 3 months after hemoglobin normalization (Mahan et al., 2012). Regarding efficiency and adverse effects, intermittent dosage and low dosage therapies have been also studied with promising results, with the latter being recommended by the WHO for developing countries (Pasricha et al., 2010; Stoltzfus, Dreyfuss, & Organization, 1998).

The prevalence of adverse gastrointestinal symptoms due to oral iron therapy ranges from 10 to 20% and is more frequent when iron is taken in an empty stomach to maximize its absorption (Mahan et al., 2012; Rimon et al., 2005). Several forms of iron are available in the market (Table 6), although the most common preparations include iron sulphate, iron gluconate or iron fumarate (Alleyne, Horne, & Miller, 2008; Macdougall, 1999). Despite of the different molecular weights of the formulations, when given at equivalent dosages of elemental iron these have similar tolerability and efficacy (Pasricha et al., 2010). Adverse side effects of oral iron therapy are mainly gastrointestinal and include nausea, epigastric distention and discomfort, heartburn, diarrhea or constipation. In patients suffering from these side effects, dose reduction, interval alteration and changes in iron preparations are proposed (Alleyne et al., 2008; Susan F. Clark, 2008; Mahan et al., 2012).

Parenteral iron administration can be used in patients who do not tolerate oral iron therapy, and/or do not comply with the prescribed treatment, have higher requirements due to bleeding or hemodialysis, or do not absorb iron secondarily to gastrointestinal disease or gastric resection (Susan F. Clark, 2008; Macdougall, 1999; Mahan et al., 2012). Parenteral iron therapy is a second line of treatment since the rise in hemoglobin levels is not faster than the one observed in oral therapies. Also, it is painful, expensive and can cause anaphylactic reactions and

adverse drugs reactions (Susan F. Clark, 2008; Goddard et al., 2000). Similar to oral iron administration, there are several formulations for parenteral iron administration which can be administered intravenously or intramuscularly (Table 6) (Alleyne et al., 2008; Susan F. Clark, 2008; Macdougall, 1999; Pasricha et al., 2010).

At a population level, several strategies can be used, alone or in combination, to prevent and correct iron deficiency. The main strategies used include (i) nutritional education with dietary modification or diversification, (ii) iron supplementation, and (iii) iron fortification of foods (Gera, Sachdev, & Boy, 2012; Zimmermann & Hurrell, 2007). Despite of all these strategies being effective, with some having an added beneficial indirect effect on problems such as malaria and helminth control or delayed clamping of the umbilical cord (Miller, 2013; Stoltzfus et al., 1998), here we will focus on food fortification.

Table 6 – Common iron compounds used in iron deficiency treatment and prevention. Adapted from Dary & Hurrell, 2006; Macdougall, 1999

Oral preparation	Parenteral preparations	Food fortificants	
Ferrous sulfate	Iron dextran	Ferrous sulfate	Ferrous fumarate
Ferrous fumarate	Iron dextrin	Ferrous gluconate	Ferrous succinate
Ferrous gluconate	Iron hydroxisacharate	Ferrous lactate	Ferric saccharate
Ferrous succinate	Iron sodium gluconate	Ferrous bisglycinate	Ferric orthophosphate
Iron polymaltose	Iron polymaltose	Ferric ammonium citrate	Ferric pyrophosphate
Polysaccharide-iron complex	Iron sorbitol citrate	Sodium iron EDTA	Elemental iron

Iron fortification is the central component of the efforts to control and prevent iron deficiency (Miller, 2013). It is also the most practical sustainable and cost-effective long term solution at the population level (Zimmermann & Hurrell, 2007). The average annual costs/person of food fortification can range from $0.06 (€0.04) in Southeast Asian sub-region to $0.15 (€0.11) in the European sub-region according to the WHO classification, indicating that the cost are not high when observed the beneficial effects obtained (Horton, 2006; Laxminarayan et al., 2006). Despite of the cost-effectiveness of iron fortification, iron is the most challenging nutrient to be used as a supplement. The iron compounds with the higher bioavailability are also the ones that strongly interact with food constituents and produce undesirable organoleptic alterations. However, there are several options (Table 6), with different properties that can be suitable depending on the food vehicle (Dary & Hurrell, 2006). Thus, success of iron fortification of foods is dependent of the food vehicle, the duration of storage, the fortificant and the composition of the food [for example: highly soluble (low molecular weight), high-affinity complexes such as Na-

Fe-EDTA are suitable for food vehicles rich in phityc acid and for long shelf-life foods] (Dary & Hurrell, 2006; Mackenzie & Garrick, 2005).

The effects of iron food fortification are unequivocal and beneficial for nutritional status and hemoglobin concentration, suggesting that iron fortification of foods is effective and can be a viable public health option to combat iron deficiency (Gera et al., 2012). Still, once again, the main body of literature assessing the effect of iron fortification is focused on children and we found no literature regarding the health impacts of iron fortification in older adults. Regarding children, there is no evidence that iron fortification improves growth (Sachdev, Gera, & Nestel, 2006) or resistance to infections (Gera & Sachdev, 2002), albeit a significant moderated positive effect of iron fortification was observed in mental development (Sachdev, Gera, & Nestel, 2005).

2. Research objectives

Here we investigate whether a low iron status is associated with decrements in cognitive and/or physical performance in senior individuals. This derived from lines of evidence in other population groups (strata) showing that in children, women of child bearing age and pregnant women, and/or in cancer patients, there is an association between the iron status and the cognitive and physical performances. Furthermore, the biochemical knowledge about the importance of iron to muscle proteins and to the oxygen transport system, particularly to the brain, which has high iron requirements, provides the molecular/biological basis for the research goals.

Specifically, in a cohort of older individuals, using a cross-sectional analysis followed by a quasi-experimental (intervention) study, the objectives were to:

1. Correlate cognition, mood, functional ability and physical performance with iron status (cross-sectional analysis);

2. Assess the effectiveness and usefulness of low dosage of iron, through fortified foods (usually recommended for the prevention of nutritional deficiencies), to normalize iron status of aged individuals (intervention branch);

3. Investigate whether cognition, mood, functional ability and physical performance could be ameliorated or normalized by the treatment of ID.

3. Material and methods

3.1.　　　Subjects and procedures

The n=162 participants enrolled in the study were recruited from health primary care centers (Braga and Guimarães/Vizela), internal medicine outpatient care (Hospital de Braga) and emergency department visits (Hospital de Braga). Participants were older community-dwelling individuals aged 55 years or more, males and females, with a general good health status, integrated in the community and with independency to perform the activities of the daily living. Exclusion criteria at enrollment included incapacity and/or inability to attend the assessment sessions, cognitive impairment, dementia diagnosis and/or inability to understand informed consent, disorders of the central nervous system and/or overt thyroid pathology. The cohort was established in accordance with the principles expressed in the Declaration of Helsinki and the work approved by the national ethical committee (Comissão Nacional de Protecção de Dados) and by local ethics review boards. All the participants gave voluntary informed signed consent.

Blood samples were collected by venipuncture and a baseline characterization was conducted which comprised a clinical interview, nutritional status and body composition assessment and evaluation of physical functional ability (Moment A1, Figure 4). From the participants that were initially characterized, a subset (with and without ID) was selected for enrollment in the intervention study (n=41). Selection of participants was based on the results of the blood analysis [ID was defined as low serum FT level or two biomarkers (MCV, MCHC, RDW, Fe, TF sat., TIBC, sTFR sTFr-LogFT index)] indicating ID as described elsewhere (L. E. Murray-Kolb & Beard, 2007; Rimon et al., 2005; Yavuz et al., 2012); the cutoff points used were: MCV<80 fL, MCHC<32 g/dL, RDW>14%, Fe<71 µg/dL, FT<45 ng/mL, TF sat.<20%, TIBC≥360 µg/dL, sTFR>1.76 mg/dL and sTFr-LogFT index>1.5. Exclusion criteria at enrollment for the intervention branch included: severe anemia (hemoglobin<9 mg/dL), chronic renal disease (previously diagnosed or creatinine >2.5 mg/dL); celiac or Crohn disease; ongoing treatment for ID; TF saturation above 55%; and/or premenopausal women. Individuals that met the inclusion criteria for the iron fortification branch were invited to consume an iron fortified desert at breakfast (daily) or assigned to a non-iron fortification intervention branch and submitted to all the procedures that were performed in the iron fortification branch, with the exception of the daily intake of the iron fortified desert. A similar amount of individuals with iron deficiency (with or without anemia) was allocated to each branch (with or without iron-fortified food). For this subset of individuals [intervention with fortification (cases, n=12) and non-fortification (non

supplemented, n=10)] an additional assessment was performed in order to obtain a more detailed characterization regarding neurocognitive/psychological dimensions, functional ability and physical performance (Moment A2, Figure 4).

The intervention had a duration of 14 weeks (+/- 2 weeks), after which a final endpoint assessment was performed (Moment B, Figure 4), with monitoring midpoints distributed throughout the study to guarantee and motivate the continued desert intake and/or participation in the study. The endpoint assessment consisted in a blood sample collection, nutritional status and body composition assessment, evaluation of physical performance and functional abilities, and neurocognitive/psychological assessment.

Overall assessments were conducted at the local health care centers or in the Clinical Academic Center – Braga (CCA-B).
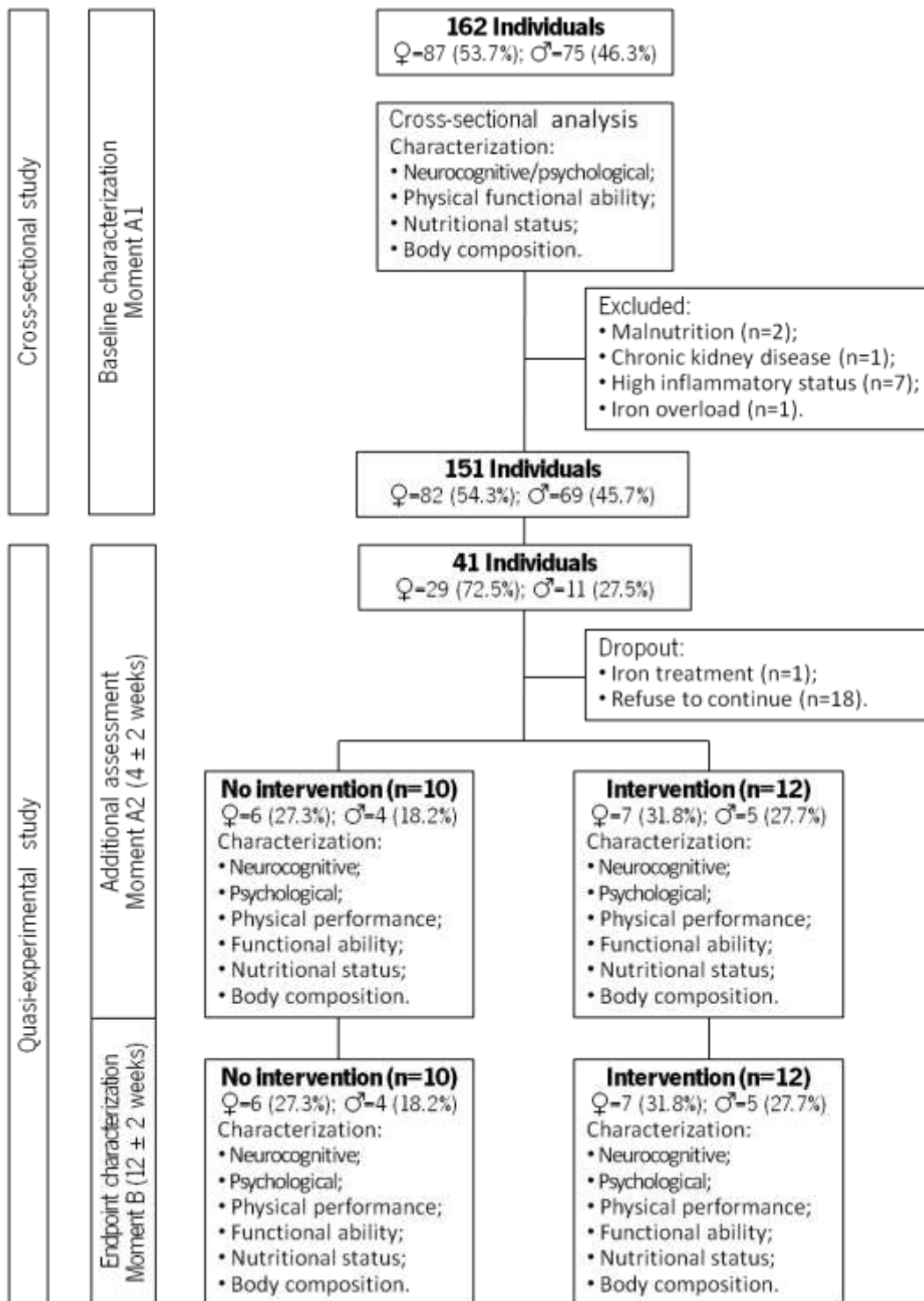
Figure 4 – Flow Diagram of the study.

3.2. Iron food fortification

Participants in the quasi-experimental intervention study that were assigned to the iron fortification branch consumed an iron and ascorbic acid fortified fruit based desert. Theoretical development of the deserts was performed by our team and accounted for the daily

requirements (recommended dietary allowance and upper limit for individuals with 50 years of age or older) of iron and ascorbic acid according to the data available from the Food and Nutrition Board of Institute of Medicine (IOM, 2000, 2001). Iron and ascorbic acid supplements were chosen in accordance with the "*Guidelines on food fortification with micronutrients*" (Dary & Hurrell, 2006) of the WHO and the FAO and based on bioavailability and physical characteristics of the food vehicle. Briefly, it was defined that each daily dose should contain 15 mg of elemental iron in the form of ethylenediaminetetraacetic acid ferric sodium salt [120 mg of $Na(Fe^{3+})EDTA$ with 12.5% mass of iron] and 90 mg of vitamin C (ascorbic acid – E300) corresponding to a 2:6 mass ratio (iron:ascorbic acid) described to increase iron absorption from foods 2- to 3-fold (Dary & Hurrell, 2006; Hurrell, 2002). Technical development was handled by Frutech (Frulact SGPS; Maia; Portugal) and sensory analysis was performed by Frutech technicians and our team. Two deserts of different flavors were chosen (apple and peach) and produced by Frulact Nutrição (Frulact Nutrição, Lda; Maia, Portugal). Apple and peach puree (50% of mass) were used as a major ingredient. After shelf life tests, bromatological analysis were performed by an independent laboratory (Silliker Portugal, S.A.; V. N. Gaia, Portugal), yielding 14.8 mg of iron and 158 mg of ascorbic acid *per* 120 g (daily dose) of apple desert and 13.26 mg of iron and 168 mg of ascorbic acid *per* 120 g (daily dose) of peach-based desert. Since ascorbic acid is easily lost during processing and storage, the higher amounts of ascorbic acid than the foreseen ensured that after storage losses and in the end of the shelf life a sufficient amount was still present.

3.3.   Laboratory analyses

Blood samples were collected by venipuncture before the assessments and immediately sent to the Pathology Laboratory at the Hospital de Braga for analyses. Blood cells count and hemogram were performed using certified standardized methods and comprised RBC (10^{12}/L), hemoglobin (mg/dL), hematocrit (%), MCV (fL), MCH (pg), MCHC (g/dL) and RDW (%). Serum iron (Fe; µg/dL) and TIBC (µg/dL) were determined by a colorimetric method using Dimension Vista System Flex reagent cartridge (Siemens, Frimley, Camberly, UK). High sensitive C-reactive protein (hsCRP; mg/dL), TF (mg/dL), FT (ng/mL) and serum concentration of sTFR (mg/dL) were measured by chemiluminescent immunoassays. Dimension Vista System Flex reagent cartridge (Siemens, Frimley, Camberly, UK) was used to measure TF, FT and sTFr; the BN* II and BN ProSpec System (Siemens, Frimley, Camberly, UK) was used to measure hsCRP. All determinations were performed following the manufacturers' instructions. Detection limits for

hsCRP, Fe, TIBC, TF, FT and sTFR are 0.175 mg/dL, 5 μg/dL, 8 μg/dL, 8.75 mg/dL, 0.5 ng/mL and 0.017 mg/L respectably. Serum TF saturation (%) was calculated as a percentage of serum total iron divided by TIBC. sTFR-LogFT index was calculated by sTFR divided by the logarithm of FT. Body iron was calculated using the Cook algorithm (Cook et al., 2003) as follows: body iron (mg/kg) = -[log(sTFR*1000/FT) − 2.8229/0.1207].

3.4. Neurocognitive/psychological assessment

Tests were selected to provide mood and cognitive (general cognitive status and executive and memory functions) profiles, as previously reported (Santos et al., 2013). The neurocognitive/psychological characterization was performed by a team of trained psychologists following the instructions provided in the Standard Operating Procedures (SOP) manual, which is a written manual were all the procedures for test application are described and is used as guideline, ensuring the standardization of the test application and results comparability.

3.4.1. Baseline characterization

The following cognitive measures were used: global cognitive status was assessed with the MMSE (Folstein, Folstein, & McHugh, 1975) and the Montreal cognitive assessment (MOCA) (Nasreddine et al., 2005); short-term verbal memory with the digit span forward test (DS forward; subtest of the Wechsler adult intelligence test WAIS III), verbal working memory with the digit span backward test (DS backward; subtest of the Wechsler adult intelligence test WAIS III) and digit span total score (DS total; calculated by the summation of DS forward and DS backward) (Strauss, 2006); multiple trial verbal learning and memory with the selective reminding test [SRT – List A; parameters: consistent long term retrieval (CLTR), long term storage (LTS), delayed recall (DR) and intrusions] (Buschke, Sliwinski, Kuslansky, & Lipton, 1995), and the consortium to establish a registry for Alzheimer's disease-word list test [CERAD, parameters: Total hits and DR hits] (Morris et al., 1989); response inhibition/cognitive flexibility with the Stroop color and word test [Stroop, parameters: Words (W), Colors (C) and Words/colors (W&C)] (Strauss, 2006). The geriatric depression scale (GDS, long-version) (Yesavage et al., 1983) was used for depressive mood evaluation.

3.4.2.   Additional assessment

Additionally to the neurocognitive/psychological characterization preformed in moment A1, the Boston naming test 15-item version (BNT-15) test (Lansing, Ivnik, Cullum, & Randolph, 1999; Mack, Freed, Williams, & Henderson, 1992) and the controlled oral word association test F-A-S (COWAT-FAS, FAS – Admissible) (Lezak, 2004) were performed in moment A2 for the subjects enrolled in the intervention study for additional neurocognitive characterization,. Also, further measures were added to the psychological assessment, namely: Beck anxiety inventory (BAI) (Beck & Steer, 1990), Beck depression inventory (BDI) (Beck, Ward, & Mendelson, 1961) and the perceived stress scale (PSS) (Cohen, Kamarck, & Mermelstein, 1983).

3.4.3.   Endpoint characterization

At the endpoint assessment, since several neurocognitive tests do not possess test-retest validity, the Addenbrooke's cognitive examination-revised (ACER) (Mioshi, Dawson, Mitchell, Arnold, & Hodges, 2006) was used to assess general cognitive status and dimension specific scores. The SRT [SRT – List B; parameters: CLTR, LTS, DR and intrusions] and a different subset of images of the BNT-15 were also used; despite of differences in the lists and the subset of images, the results of SRT and BNT-15 from baseline characterization and endpoint characterization were directly comparable which avoids learning interference. Neuropsychological assessment was performed using depression, anxiety and stress scales – 21 items (DASS-21) (Lovibond & Lovibond, 1995).

3.5.   Assessment of functional ability and physical performance

The questionnaire of functional ability (QoFA) was the only instrument used to assess functional ability at the baseline characterization (Moment A1). All the remaining tests, as nest described, were used in the additional characterization (Moment A2) and in the endpoint assessment (Moment B). Instructions provided in the SOP manual were followed.

3.5.1.   Questionnaire of functional ability

The QoFA is a scale that measures the ability of older individuals to perform physical activities of daily living (Avlund, Kreiner, & Schultz-Larsen, 1996). The instrument is composed of 16 questions, for each question two more are made regarding tiredness and help needed to

perform the task. The instrument is composed of 3 subscales in relation to tiredness, (i) mobility tiredness, (ii) lower limb tiredness and (iii) upper limb tiredness, and 2 subscales in relation to dependency, (iv) mobility help and (v) physical activities of daily living help. Higher values in each subscale represent higher functionality. The instrument was applied after a brief explanation of its structure and aims.

3.5.2. Hand grip strength

The Jamar hand dynamometer (Lafayette Instrument Company, USA) was used. This instrument is the most widely cited in the literature and accepted as the gold standard by which other dynamometers are evaluated. The dial reads force in kg with markings at intervals of 2 kg, allowing assessment to the nearest kg. Here we used an adaptation of the protocol of the American Society of Hand Therapists that was recently proposed (Roberts et al., 2011). The same procedure was used for left hand and three measurements for each hand, alternating sides, were taken. The recorded result was always the highest results in each trial. Hand dominance was recorded (right, left or ambidextrous – people who can genuinely write with both hands). The best of the six grip strength measurements was used for statistical analyses.

3.5.3. 6-m timed walk

The 6-m timed walk (6MTW) is an adaptation of the 10 m timed walk which is well established for use in assessment of patients with stroke. The validity and reliability of the 6MTW to assess walking ability has been demonstrated (Lam, Lau, Chan, & Sykes, 2010). Gait speed (m/min) and cadence (steps/min) was calculated from the data collected.

3.5.4. Tinetti evaluation

The performance oriented mobility assessment (POMA) is a simple and easy to administer test used to evaluate gait and balance (Tinetti, 1986). The test was originally proposed to assess the risk of falling in older individuals. It is composed by 2 scales, one with 9 parameters for the assessment of static balance and a second with 10 parameters which assess gait balance.

3.6.   Nutritional status and body composition assessment

All the procedures described in this section were performed at the baseline characterization (Moment A1) and at the endpoint assessment (Moment B).

3.6.1.   Mini nutritional assessment

The mini nutritional assessment (MNA) is a screening tool that helps to identify aged malnourished people or at risk of malnutrition before the development of alterations in weight or in serum proteins (Kondrup, Allison, Elia, Vellas, & Plauth, 2003). The full MNA is composed of 2 parts: (i) 'Screening' to identify malnourished people or at risk of malnutrition, and (ii) 'Assessment' to allow for the determination of potential causes of malnutrition. All the questions of the MNA were applied in a face-to-face interview; data on body mass index (BMI), mid arm circumference and calf circumference were obtained during the anthropometric characterization. The malnutrition indicator score was calculated by the summation of the score obtained in the screening and assessment, which result in a classification in 3 categories: malnutrition, at risk of malnutrition and normal nutritional status.

3.6.2.   Weight and bioelectrical impedance analysis

Weight and relative body fat mass (%BF) were measured with the participants wearing light wear using a Tanita® BF 350 Body Composition Analyzer (Tanita Corporation, Tokyo, Japan), which uses the foot-to-foot bioelectrical impedance analysis (BIA)(Yanovski, Hubbard, Heymsfield, & Lukaski, 1996) to estimate %BF. Participants were invited to stand on the scale without support and with the weight equal distributed in both legs. The output variables were calculated according to the manufacture's embedded software.

3.6.3.   Anthropometric characterization

Anthropometric characterization consisted in the measurements of height, waist circumference, hip circumference, mid arm circumference, calf circumference; and the thickness of: triceps skinfold, biceps skinfold, subscapular skinfold and suprailiac skinfold. All measurements were taken in accordance with the international standards for anthropometric assessment (A. Stewart, Marfell-Jones, Olds, & Ridder, 2011) from the International Society for

the Advancement of Kinanthropometry, in triplicate and the mean value used to calculate the derivate indexes.

Briefly, height was measured without shoes using a stand-alone stadiometer Seca® 217 (Seca GmBH & Co Kg, Hamburg, Germany). Circumferences were measured using an ergonomic and smooth measuring tape Seca® 201 (Seca GmBH & Co Kg, Hamburg, Germany) and only the minimal amount of pressure was made warranting that the tape was not excessively indented to the skin. Waist circumference was measured at the mid-point between the lower costal (10th rib) border and the iliac crest in the end of a normal expiration (end tidal). Hip circumference was taken in a horizontal plane at the level of the greatest posterior protuberance of the buttocks, which usually corresponds anteriorly to the level of the symphysis. Mid arm circumference was measured at the mid-point between acromial surface of the scapula and the olecranon process perpendicular to the longest axis of the arm. Calf circumference was measured with the subjects seated and the knee bent at 90° angle, the tape was slipped in order to measure the maximum girth of the calf perpendicular to the longest axis of the leg. BMI was calculated according to the weight (kg)/height (m)^2 ratio.

Skinfolds thickness was measured using the Lipotool (Liposoft 2008 & Adipsmeter V0) (Amaral et al., 2011). All participants were in the anatomical position and relaxed, the measurements were taken in the right side of the body. Triceps skinfold thickness was measured in the arm at the mid-point between acromial surface of the scapula and the olecranon process in the most posterior site of the triceps when viewed from the side. Biceps skinfold thickness was measured at the same level of the triceps skinfold in the most anterior site of the biceps when viewed from the side. Triceps and biceps skinfolds were measured parallel to the longest axis of the arm. Subscapular skinfold thickness was measured at the point 2 cm from the subscapular point in a line 45° laterally downward determined by the natural fold of the skin. Suprailiac skinfold was taken when the right arm of the participant was abducted to the horizontal. This skinfold was measured at the line that defines the anterior-posterior division and immediately superior to the iliac crest. The direction of the fold runs slightly downwards anteriorly as determined by the natural fold of the skin. The mean value of the skinfold thickness was used to estimate body density taking into account that for individuals with age comprised between 50 and 59 it was calculated using the Durnin and Womersley (Durnin & Womersley, 1974) sex-specific equations and for older individuals the Visser et al.(Visser, Heuvel, & Deurenberg, 1994) sex-

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specific equations. %BF was calculated from body density using the Brozek *et al.* (Brožek, Grande, Anderson, & Keys, 1963) (%BF-Brozek) equation.

3.7.   Statistical analysis

Characteristics of participants are presented in mean and standard deviation (mean; SD) for normal distributed variables and in median and interquartile range for variables with a non-normal distribution. To evaluate normal distribution of the variables, skewness and kurtosis values were calculated and the approximate normal distribution was defined for variables with absolute values of skewness below 3 and of kurtosis below 8 (Kline, 2011). Log transformations were performed to normalize the distribution of skewly distributed variables (hsCRP, FT; sTFR and sTFR-LogFT index). Independent samples t-test (for variables with normal distribution) and Mann-Whitney U test (for variables with non-normal distribution) were performed to analyze the differences in socio-demographic variables, anthropometric, psychological, neurocognitive and physical functional ability between men and women; individuals with or without ID in the cross-sectional study and in the same variables at baseline between non supplemented and supplemented in the longitudinal study. To quantify the strength of the differences, Cohen´s d was calculated as a measure of effect size (.2 is considered a small effect size, .5 a medium effect size and .8 a large effect size) (Kotrlik & Williams, 2003); in Mann-Whitney U test r was calculated and reported as the measure of effect size. Differences in categorical variables were assessed using Chi squared test and effect size reported as Phi (φ) or Cramer's V (φ_c). When assumptions for Chi-square tests on contingency tables were violated the two tailed significance level of Fisher exact test was used ($P_{(Fisher\ exact\ test;\ 2\ tailed)}$).

All variables were converted into z-scores to express all variables in the same scale. Principal component analysis (PCA) was conducted to reduce the number of variables with a minimum loss of information and, therefore, reducing the number of comparisons. New component scores were obtained (using the regression method) and were used in subsequent analyses. The reliability of each component was analyzed using Cronbach's alpha. Components were considered reliable when Cronbach's alpha was higher than .6 (DeVellis, 2011). Variables not included in PCA were analyzed independently.

Binary logistic regression analysis was used to test if nutritional status (MNA) and body composition (BMI, %BF-Tanita and %BF-Brozek) were significant predictors of ID when controlled for potential confounding factors (age, gender and hsCRP) using the enter method.

Analyses of covariance (ANCOVA) were used to test differences in dependent variables (psychological, neurocognitive and physical functional ability) between individuals with or without ID, controlling for the principal confounding factors (age, education, gender and hsCRP for psychological variables; the previous plus GDS score for neurocognitive variables and dimensions; and age, BMI and hsCRP for physical functional ability dimensions). Education (school years; <4=0; ≥4=1) was converted to a dummy variable due to the high concentration of individual in the 4 years.

Hierarchical regression analysis was performed to compare different hematological dimensions (that resulted from PCA) as predictors of previously mentioned dependent variables controlling for the principal, above mentioned, confounding factors (using enter method). Interaction (mediator effect) between nutritional status and hematological dimensions was tested using a mean centered methodology and hierarchical regression analysis as previously described.

Analysis of repeated measures (general linear model – repeated measures ANOVA) was used for comparison of variables across different time-points (when repeated measures were available) during the intervention study. For non-normal distributed variables (skewly distributed) (FT, sTFR and sTFR-LogFT index) logarithmic transformation was applied allowing the use of repeated measures ANOVA, therefore data are presented as geometric mean and back-transformed 95% confidence intervals. Two independent sample t-tests (pre- and post-intervention) were used to compare data from different variables measuring the same construct before and after intervention (no repeated measures available).

Statistical analysis was conducted using the SPSS package v22 (IBM SPSS Statistics) and statistical significance was defined at $p < .05$ level. In the tables the most relevant significant results are highlighted in gray color.

3.8.   Team

This thesis project is based on a larger study addressing predictors of healthy cognitive aging. As such, a multidisciplinary team participates and conducts the various aspects of the study. With respect to the data presented in this thesis:

The design and supervision of the study was accomplished by Professor Joana Palha, Professor Nuno Sousa and Dr. Nadine Correia Santos.

Measurement of blood parameters was done at the Pathology Laboratory at the Hospital de Braga.

Neurocognitive and neuropsychological assessments were performed by Teresa Costa Castanho, Liliana Amorim and Pedro Silva Moreira.

Functional ability, physical performance, nutritional status evaluations and body composition assessments were performed by me. I was also responsible by data analysis, with the valuable support and insights of Professor Patricio Costa, Dr. Nadine Correia Santos and Pedro Silva Moreira, and writing of this report.

4. Results

4.1 Cross-sectional analysis: iron status correlates of cognition and physical status and performance

4.1.1 Participants characterization

The initial sample was composed by 162 individuals, from which 11 participants were excluded due to malnutrition (n=2), chronic kidney disease (n =1; creatinine >2.5 mg/dL), high inflammatory status (n=7; hsCRP> 10 mg/L) and iron overload (n=1; TF saturation>55%). After exclusion the study sample comprised 151 individuals [females, n=82 (54.3%); males, n=69 (45.7%)]. Characteristics of the participants, for the all sample and by gender are shown in Table 7. No significant differences were observed between females and males for age although, as expected and previously reported for similar samples (Santos et al., 2014), males had more years of formal education than females. No significant differences were observed in gender distribution by BMI classes or class of nutritional status.

Table 7 - Socio-demographic, anthropometric and nutritional characteristics of participants.

Variables (mean; SD)	All	Females	Males	
Socio-demographic				$t_{(df)}$; **p**; Cohen's d
Age (yrs)	66.30; 7.87	67.16; 7.66	65.29; 8.04	$1.459_{(149)}$; .147; .240
Education (school yrs)	5.19; 3.83	4.40; 3.45	6.12; 4.08	$-2.757_{(133.799)}$; .007; .460
Anthropometric				$t_{(df)}$; **p**; Cohen's d
Weight (kg) [a]	72.88; 11.69	68.77; 10.65	77.48; 11.14	$-4.762_{(140)}$; <.001; .806
Height (m) [a]	1.59; 0.09	1.53; 0.05	1.65; 0.07	$-12.568_{(140)}$; <.001; 2.128
BMI (kg/m^2) [a]	28.97; 3.86	29.48; 4.00	28.4; 3.64	$1.664_{(140)}$; .098; .282
Waist circ. (cm) [a]	95.69; 10.58	93.65; 11.18	97.97; 9.43	$-2.468_{(140)}$; .015; .418
Hip circ. (cm) [a]	101.46; 7.73	102.98; 8.15	99.75; 6.90	$2.534_{(140)}$; .012; .429
%BF-BIA (%) [b]	32.70; 7.85	37.27; 5.49	27.41; 6.80	$9.427_{(136)}$; <.001; 1.621
%BF-Brozek (%) [a]	34.54; 6.99	40.09; 2.99	28.33; 4.51	$18.49_{(140)}$; <.001; 3.130
BMI class (n; %)				$\chi^2_{(df)}$; **p**; φ_c
Normal	23; 16.20	12; 8.45	11; 7.75	$2.744_{(2)}$; .250; .139
Overweight	67; 47.18	31; 21.83	36; 25.35	
Obesity	52; 36.62	32; 22.54	20; 14.08	
Nutritional status (n; %)				$\chi^2_{(df)}$; **p**; φ
Risk of malnutrition	20; 14.08	14; 9.86	6; 4.23	$2.758_{(1)}$; .146; .139
Normal	122; 85.92	61; 42.96	61; 42.96	

[a] n=142 [females=75 (52.82%). males=67 (47.18%)]; [b] n=138 [females=74 (53.62%). males=64 (46.38%)]. Data presented for all sample and by gender. Statistical test performed between gender.

Descriptive statistics for neuropsychological and cognitive variables are presented in Table 8. Females scored significantly higher in neuropsychological variables indicative of higher depressive mood and perceived stress, and significantly lower in all neurocognitive variables Stroop W, Stroop W&C, MMSE and MOCA.

From the PCA, two cognitive dimensions were obtained based on the neurocognitive variables (with less than 10% of missing values), termed: (i) executive dimension, and (ii) memory dimension (Figure 5). The executive dimension (executive functioning, Cronbach's alpha: 0.880) was composed of the Stroop (W, C and W&C) and DS (backward and total) parameters. The variables DS forward and MMSE total score had low communalities in the solution and were excluded from the final model. The memory dimension (memory function, Cronbach's alpha: 0.933) was composed of the SRT (CLTR, LTS and DR) and the CERAD (total hits and DR) parameters. Intrusions in SRT were excluded due to low communalities. Significant differences were observed in the executive dimension with females scoring lower, and no differences were observed in the memory dimension (Table 8, Figure 6).
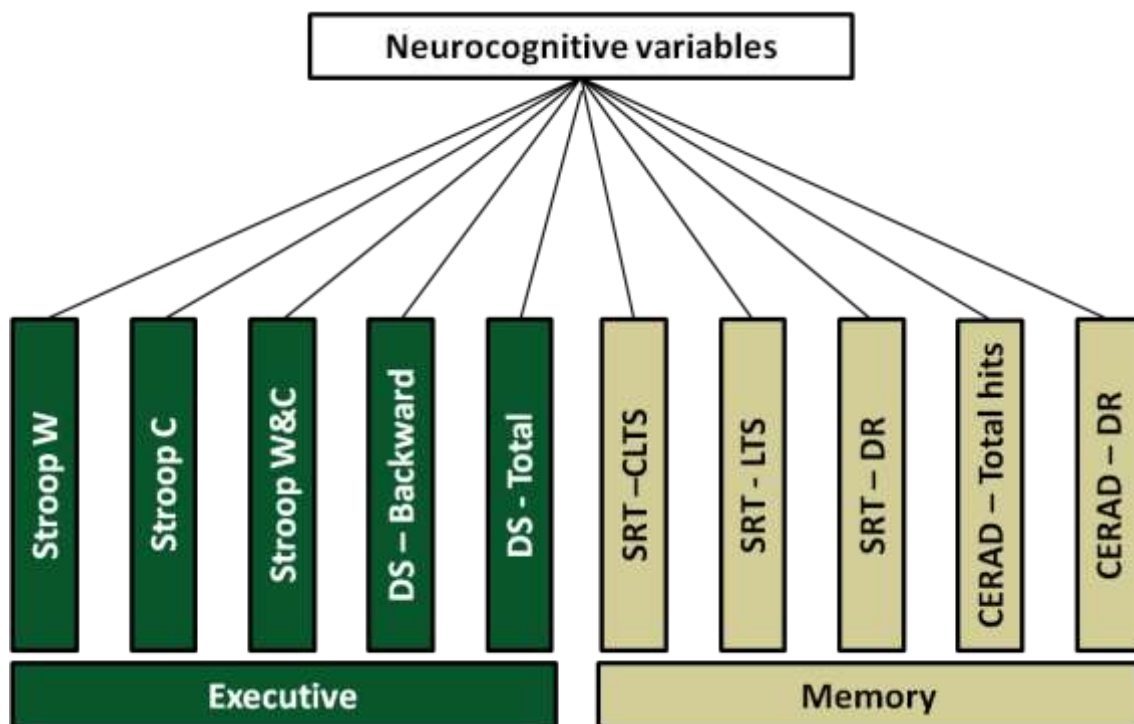
Figure 5 – Diagram of neurocognitive dimensions formation by PCA.

Table 8 - Neuropsychological and neurocognitive variables in all sample and by gender.

Variables (mean; SD)	All	Females	Males	
Psychological				$t_{(df)}$*; p*; **Cohen's d**
GDS	11.25; 6.36	13.23; 6.10	8.88; 5.88	$4.437_{(149)}$; <.001; .730
PSS [a]	20.37; 7.52	22.73; 7.67	17.77; 6.46	$4.093_{(137)}$; <.001; .700
Neurocognitive				$t_{(df)}$*; p*; **Cohen's d**
DS – Forward	7.39; 2.24	6.84; 1.99	8.04; 2.35	$.825_{(149)}$; .411; .136
DS – Backward	4.16; 2.32	3.60; 1.91	4.81; 2.58	$.825_{(149)}$; .410; .136
DS – Total	11.55; 4.15	10.44; 3.43	12.86; 4.55	$.733_{(149)}$; .465; .120
Stroop – W [b]	65.29; 21.70	58.04; 21.23	73.73; 19.17	$-4.640_{(143)}$; <.001; .778
Stroop – C [c]	48.34; 14.79	46.99; 14.57	49.90; 15.00	$-1.196_{(146)}$; .234; .198
Stroop – W&C [c]	29.72; 12.99	27.44; 11.61	32.33; 14.04	$-2.289_{(132.34)}$; .024; .385
SRT – LTS	25.34; 13.77	23.43; 13.22	27.62; 14.15	$-1.881_{(149)}$; .062; .309
SRT – CLTR	15.58; 12.81	14.8; 12.46	16.51; 13.25	$-.813_{(149)}$; .418; .134
SRT – DR [d]	5.65; 2.85	5.55; 2.78	5.78; 2.94	$-.497_{(140)}$; .620; .084
CERAD – Total hits [e]	17.59; 4.93	17.24; 5.15	17.99; 4.68	$-.898_{(140)}$; .371; .152
CERAD – DR [e]	5.77; 2.40	5.48; 2.42	6.09; 2.37	$-1.515_{(140)}$; .132; .256
MMSE	26.52; 3.27	25.67; 3.58	27.52; 2.54	$-3.708_{(145.03)}$; <.001; .593
MOCA [f]	18.06; 5.13	16.98; 5.17	19.45; 4.78	$-2.644_{(115)}$; .009; .497
Cognitive dimensions				$t_{(df)}$*; p*; **Cohen's d**
Executive [g]	0; 1.00	-.26; .91	.30; 1.02	$-3.505_{(141)}$; .001; .592
Memory [h]	0; 1.00	-.10; 1.00	.11; 1.00	$-1.259_{(131)}$; .210; .220

[a] n=139 [females=73 (52.52%). males=66 (47.48%)]; [b] n=145 [females=78 (53.79%). males=67 (46.21%)]; [c] n=148 [females=79 (53.38%). males=69 (46.62%)]; [d] n=142 [females=77 (54.23%). males=65 (45.77%)]; [e] n=142 [females=75 (52.82%). males=67 (47.18%)]; [f] n=117 [females=66 (56.41%). males=51 (43.59%)]; [g] n=143 [females=76 (53.15%). males=67 (46.85%)]; [h] n=133 [females=70 (52.63%). males=63 (47.37%)]. Data presented for all sample and by gender. Statistical test performed between gender.
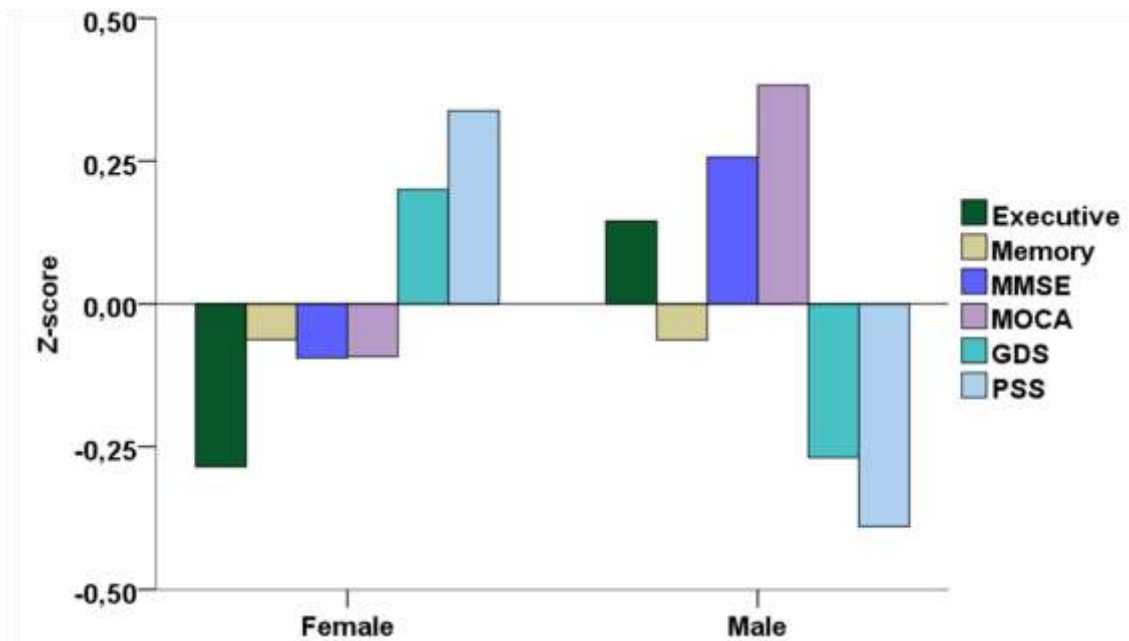
Figure 6 – Mean value of neurocognitive and neurpsychological variables/dimensions by gender.

Two dimensions were obtained from the subscales of the QoFA: (i) functional-T dimension (functional tiredness, Cronbach's alpha: .763), composed by mobility tiredness, lower limb tiredness and upper limb tiredness subscales; and (ii) functional-H dimension (functional help, Cronbach's alpha: .690), composed by mobility help and PADL help (Figure 7). Similar to the variables of origin, higher scores represent higher functionality, so higher scores on functional-T represent less tiredness and higher scores on functional-H represent less need of help. In all physical functional variables sub-scales and dimensions (Table 9, Figure 8) females scored significantly lower than males.
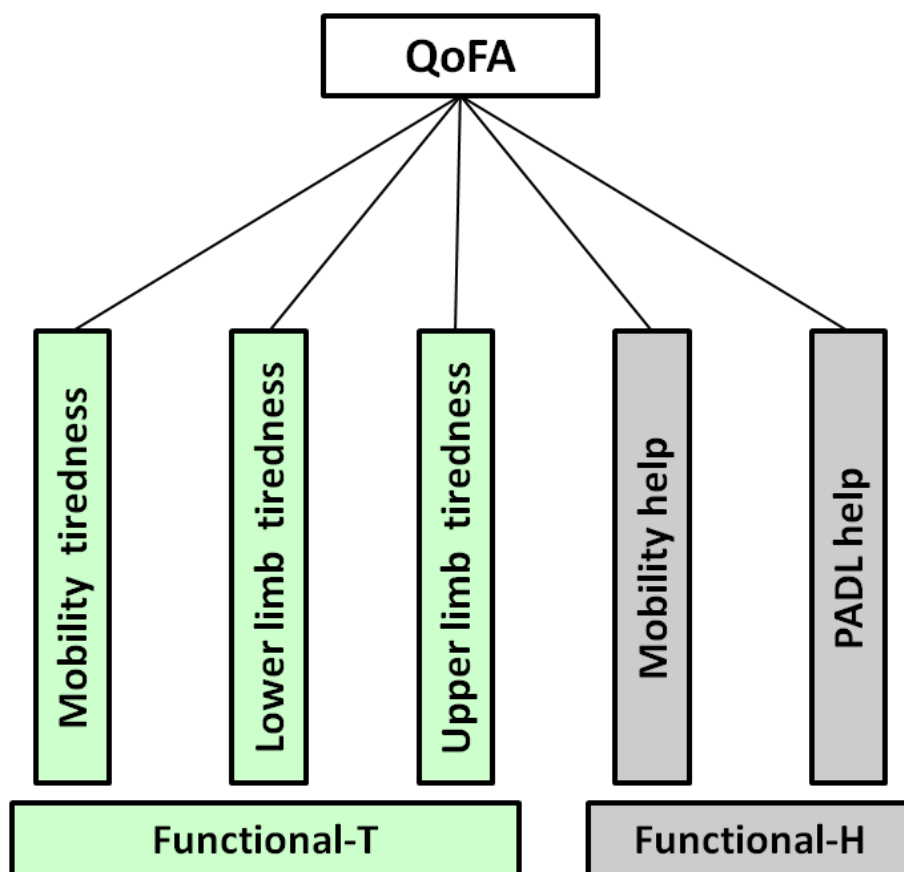
Figure 7 – Representation of functional dimension obtained by PCA.

Table 9 - Physical functional ability sub-scales and functional dimensions.

Variables (mean; SD)	All	Females	Males	
Functional ability – QoFA [a]				$t_{(df)}$; **_p_; Cohen's d**
Mobility tiredness	4.45; 1.94	3.77; 2.00	5.26; 1.52	$-5.191_{(146.19)}$; <.001; .838
Lower limb tiredness	4.24; 1.13	3.90; 1.29	4.64; .75	$-4.351_{(131.4)}$; <.001; .689
Upper limb tiredness	3.76; .77	3.57; 1.00	3.99; .12	$-3.73_{(82.72)}$; <.001; .569
Mobility help	11.05; 2.06	10.59; 2.48	11.59; 1.22	$-3.211_{(120.32)}$; .002; .504
PADL help	18.59; 2.87	17.98; 3.41	19.3; 1.84	$-3.025_{(126.62)}$; .003; .477
Functional components [a]				$t_{(df)}$; **_p_; Cohen's d**
Functional tiredness	0; 1.00	-.37; 1.14	.43; .57	$-5.529_{(122.2)}$; <.001; .869
Functional help	0; 1.00	-.25; 1.18	.29; .62	$-3.600_{(124.31)}$; <.001; .567

[a] n=150 [females=81 (54%); males=69 (46%)]. Data presented for all sample and by gender. Statistical test performed between gender.
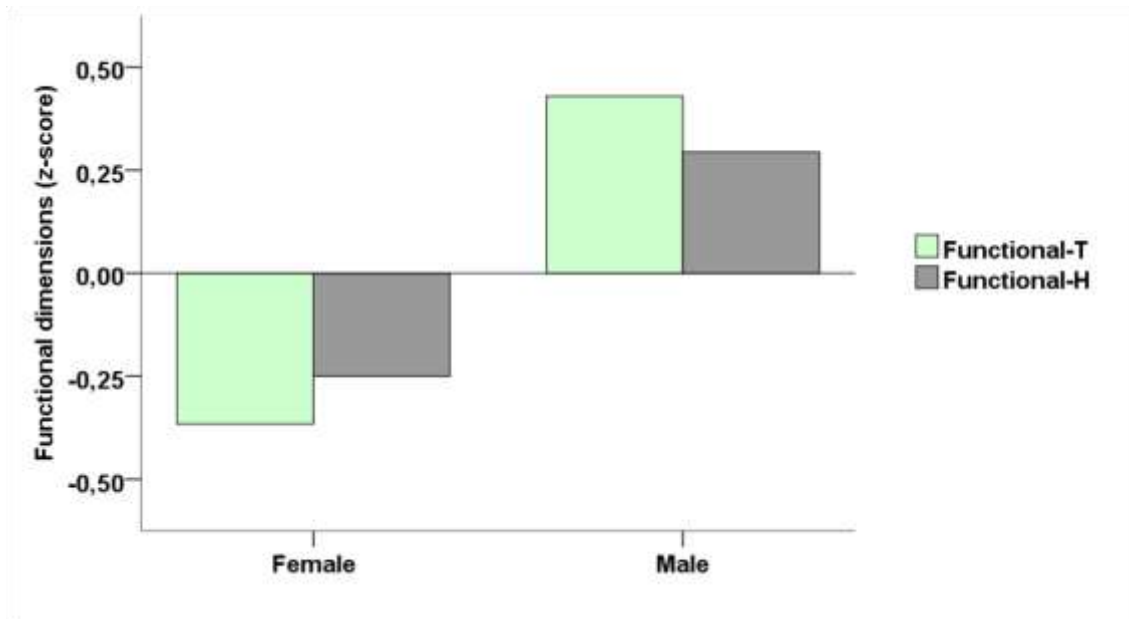
Figure 8 – Distribution of functional dimensions by gender.

Hematologic variables are shown in Table 10. The hsCRP was measured to assess the inflammatory status of participants and no significant differences were observed between males and females. With exception for MCV and RDW, all the red cells indices and iron biomarkers variables were significantly different, with the direction of difference indicating a lower iron status of females (iron biomarkers: lower Fe, FT, TF Sat.; sTFR, sTFr-LogFT index and Body iron; higher TF and TIBC).

Similar to previously described (L. E. Murray-Kolb & Beard, 2007), dimensions of hematologic variables were obtained (here using PCA). For these analyses, log-transformation of non-normal distributed variables was used and posteriorly transformed into z-scores (FT, sTFR and sTFr-LogFT index). In some cases z-scores of variables were inverted (multiplied by -1) in order to make higher values represent higher iron status (RDW, TF, TIBC, sTFR and sTFr-LogFT index). Five components were obtained using the hematologic factors: four of them using the same factors described by Murray-Kolb *et al.*(L. E. Murray-Kolb & Beard, 2007), (i) storage, (ii) transport, (iii) red cells characteristics (red cells C) and (iv) erythropoiesis; and a new one obtained from the remaining biomarkers, (iv) transport saturation (transport S) (Figure 9).
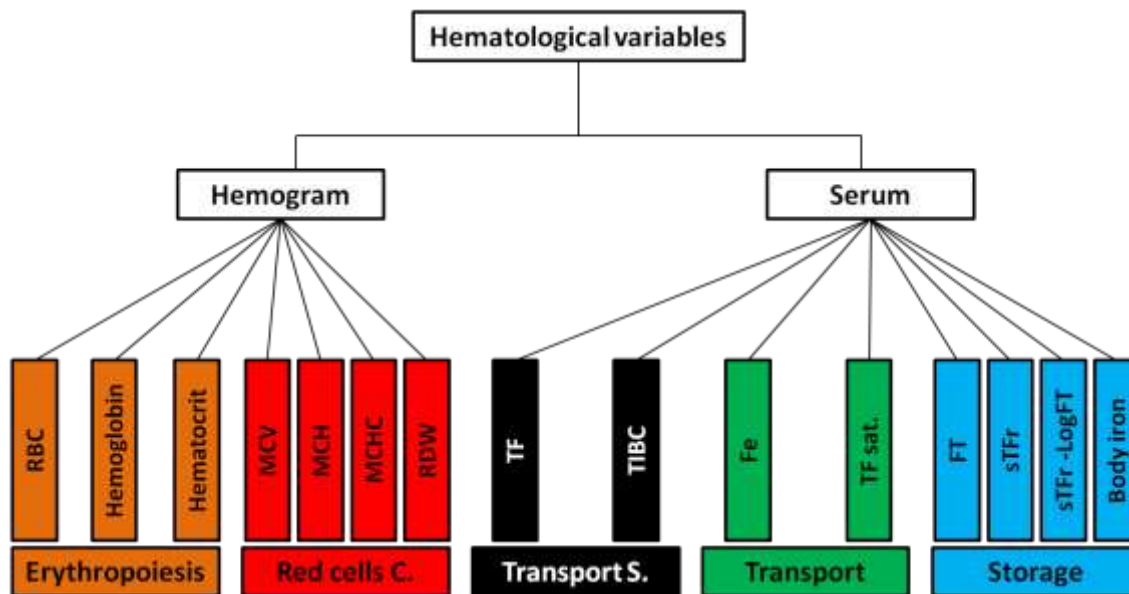
Figure 9 - Representation of hematological dimensions obtained by PCA.

Table 10 - Hematologic variables and dimensions for iron status assessment.

Variables (mean; SD)	All	Females	Males	
Inflammatory indices				$Z_{(U)}$; *p; r*
hsCRP (mg/dL) [a¥£]	1.90; 2.62	2.35; 2.80	1.60; 2.50	-1.430$_{(2378)}$; .152; .117 [£]
Red cells indices [b]				$t_{(df)}$*; p; Cohen's d*
RBC (10^{12}/L)	4.56; 0.43	4.37; 0.41	4.79; 0.35	-6.615$_{(144)}$; <.001; 1.108
Hemoglobin (mg/dL)	13.80; 1.60	13.01; 1.35	14.77; 1.34	-7.850$_{(144)}$; <.001; 1.314
Hematocrit (%)	40.37; 4.12	38.40; 3.66	42.76; 3.31	-7.478$_{(144)}$; <.001; 1.252
MCV (fL)	88.55; 4.33	87.92; 4.27	89.30; 4.31	-1.931$_{(144)}$; .056; .323
MCH (pg)	30.24; 1.84	29.78; 1.72	30.81; 1.83	-3.512$_{(144)}$; .001; .588
MCHC (g/dL)	34.15; 0.99	33.86; 0.94	34.50; 0.93	-4.123$_{(144)}$; <.001; .690
RDW (%)	13.37; 0.88	13.47; 0.91	13.25; 0.84	1.509$_{(144)}$; .134; .253
Iron biomarkers [c]				$t_{(df)}$*; p; Cohen's d*
Fe (µg/dL) [c]	93.13; 29.46	86.58; 25.05	100.65; 32.41	-2.903$_{(125.38)}$; .004; .493
TF (mg/dL) [c]	248.34; 42.63	255.74; 42.52	239.85; 41.45	2.279$_{(144)}$; .024; .381
FT (ng/mL) [c¥£]	132.00; 204.25	84.50; 129.00	221.50; 235.75	-4.840$_{(1418.5)}$; <.001; .401 [£]
TF sat. (%) [c]	27.93; 10.02	25.27; 8.45	30.97; 10.85	-3.501$_{(125.89)}$; .001; .595
TIBC (µg/dL) [c]	345.00; 55.32	354.10; 56.98	334.56; 51.82	2.156$_{(144)}$; .033; .360
sTFr (mg/L) [d¥£]	1.11; .49	1.20; .60	1.01; .38	-3.420$_{(1831)}$; .001; .281 [£]
sTFr-LogFT index [c¥£]	.55; .31	.63; .34	.45; .20	-4.880$_{(1408)}$; <.001; .404 [£]
Body iron (mg/kg) [c]	14.91; 4.68	13.37; 4.33	16.67; 4.46	-4.524$_{(144)}$; <.001; .756
Iron dimensions				$t_{(df)}$*; p; Cohen's d*
Storage [c]	0; 1.00	-.28; .93	.32; .98	-3.754$_{(144)}$; <.001; .627
Transport [c]	0; 1.00	-.25; .84	.29; 1.09	-3.276$_{(124.79)}$; .001; .557
Transport S. [c]	0; 1.00	-.17; 1.01	.20; .96	-2.241$_{(144)}$; .027; .374
Red cells C. [b]	0; 1.00	-.25; .97	.30; .96	-3.429$_{(144)}$; .001; .574
Erythropoiesis [b]	0; 1.00	-.49; .88	.59; .80	-7.639$_{(144)}$; <.001; 1.279

[a] n=149 [(females=81 (54.36%). males=68 (45.64%)]; [b] n=146 [(females=80 (54.79%). males=66 (45.21%)]; [c] n=146 [(females=78 (53.42%). males=68 (46.58%)]; [d] n=148 [(females=80 (54.05%). males=68 (45.95%)]; [¥] Variables not normally distributed. data presented in median and interquartile range (median. IQR); [£] Mann–Whitney U test. results presented in $Z_{(U)}$; *p; r*. Data presented for all sample and by gender. Statistical test performed between gender.
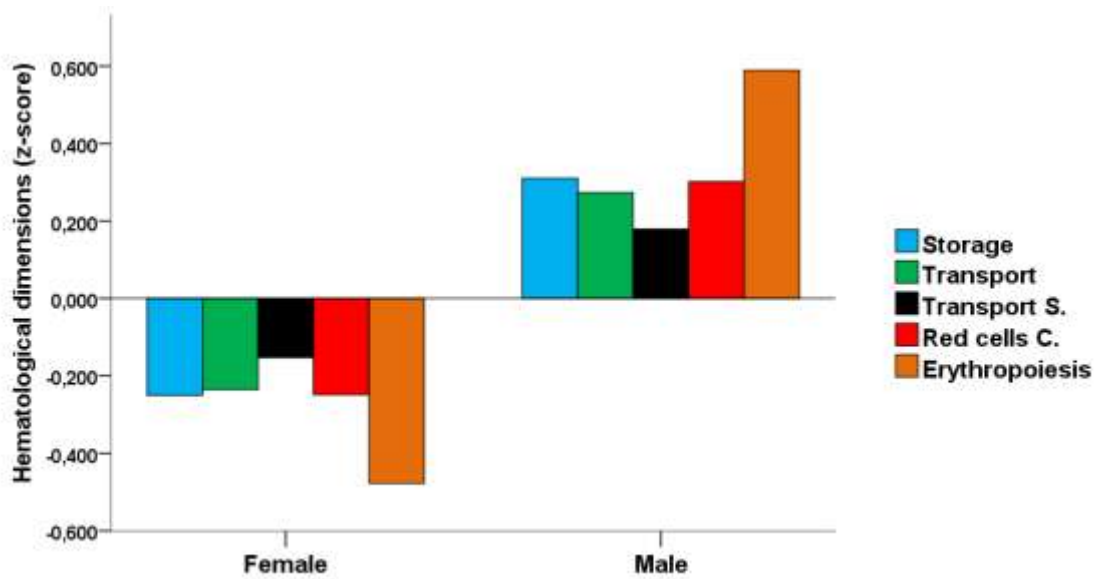
Figure 10 – Distribution of hematological dimensions by gender.

Storage dimension (storage of iron; Cronbach's alpha: .925) was composed by FT (inverted), body iron, sTFR (log-transformed and inverted) and sTFr-LogFT index (log-transformed and inverted). Transport dimension (iron transport in blood stream; Cronbach's alpha: .856) included Fe and TF sat.. Transport S. dimension (saturation of iron carrying capacity in the blood stream; Cronbach's alpha: .979) was obtained by the reduction of TF and TIBC (both inverted). Red cells C. dimension (composition, dimension and variability of red cells; Cronbach's alpha: .822) constructed with MCV, MCH, MCHC and RDW (inverted). Erythropoiesis dimension (number and relative volume of red cells and hemoglobin sufficiency; Cronbach's alpha: .967) composed by RBC, hemoglobin and hematocrit (Figure 9). Higher values in these dimensions represent higher iron status and, therefore, similar to the origin variables. Female presented a significantly lower iron status in all iron dimensions.

4.1.2 Relevance of iron deficiency for cognition, mood and physical performance

ID was defined as low serum FT level or as the presence of two biomarkers indicating ID (MCV<80 fL, MCHC<32 g/dL, RDW>14%, Fe<71 µg/dL, FT<45 ng/mL, TF sat.<20%, TIBC≥360 µg/dL, sTFR>1.76 mg/L and sTFr-LogFT index>1.5). Characterization data for iron sufficiency and ID individuals is presented in Table 11. From the 151 participants, 2 failed blood collection and therefore no data was available; from the remaining 149 participants, 44 individuals (29.53%) were classified as ID and 105 individuals (70.47%) as iron sufficiency.

Table 11 - Characteristics of participants with and without ID

Variables (mean; SD)	Iron sufficiency	ID	
Socio-demographic			$t_{(df)}$; *p*; Cohen's d
Age (yrs)	66.37; 8.26	66.09; 7.11	.197$_{(147)}$; .844; .036
Education (school yrs)	5.15; 3.87	5.18; 3.70	-.043$_{(147)}$; .966; .008
Anthropometric			$t_{(df)}$; *p*; Cohen's d
Weight (kg) [a]	73.94; 11.85	70.89; 1.73	1.426$_{(138)}$; .156; .267
Height (m) [a]	1.60; .08	1.56; .08	2.218$_{(138)}$; .028; .415
BMI (kg/m^2) [a]	28.96; 3.75	29.13; 4.17	-.236$_{(138)}$; .814; .044
Waist circ. (cm) [a]	96.58; 1.27	93.86; 11.06	1.397$_{(138)}$; .165; .261
Hip circ. (cm) [a]	101.7; 7.73	101.22; 7.80	.333$_{(138)}$; .739; .062
%BF-BIA (%) [b]	32.16; 7.76	34.24; 8.12	-1.394$_{(134)}$; .166; .266
%BF-Brozek (%) [a]	33.70; 7.21	36.60; 6.20	-2.251$_{(138)}$; .026; .421
Gender (n; %)			$\chi^2_{(df)}$; *p*; φ
Female	49; 32.89	32; 21.48	8.488$_{(1)}$; .004; -.239
Male	56; 37.58	12; 8.05	
BMI class (n; %)			$\chi^2_{(df)}$; *p*; φc
Normal	17; 12.14	5; 3.57	.756$_{(2)}$; .679; .073
Overweight	47; 33.57	19; 13.57	
Obesity	35; 25	17; 12.14	
Nutritional status (n; %)			$\chi^2_{(df)}$; *p*; φ
Risk of malnutrition	11; 7.86	8; 5.71	1.745$_{(1)}$; .277; -.112
Normal	88; 62.86	33; 23.57	

[a] n=140 [Iron sufficiency =99 (70.71%). ID=41 (29.29%)]; [b] n=136 [Iron sufficiency =97 (71.32%). ID=39 (28.68%)].

Table 12 (Figure 11) shows neuropsychological and neurocognitive variables of participants by iron status classification. No significant differences were observed except for GDS, with a higher mean value in the ID group. Data from physical functional ability (Table 13, Figure 12) did not show statistically significant differences between iron sufficiency and ID participants.

Table 12 - Neuropsychological and neurocognitive variables in iron sufficiency and ID.

Variables (mean; SD)	Iron sufficiency	ID	$t_{(df)}$; *p*; Cohen's d
Psychological			$t_{(df)}$; *p*; Cohen's d
GDS	10.47; 5.89	13.20; 6.97	$-2.449_{(147)}$; .016; .443
PSS [a]	20.23; 8.04	20.78; 6.24	$-.380_{(136)}$; .704; .072
Neurocognitive			$t_{(df)}$; *p*; Cohen's d
DS – Forward	7.70; 2.24	6.7; 2.13	$.714_{(147)}$; .476; .129
DS – Backward	4.43; 2.50	3.48; 1.66	$.711_{(147)}$; .478; .129
DS – Total	12.13; 4.37	1.18; 3.34	$.779_{(147)}$; .437; .141
Stroop – W [b]	65.85; 21.27	63.51; 22.39	$.586_{(141)}$; .559; .109
Stroop – C [c]	48.30; 15.10	47.70; 13.81	$.225_{(144)}$; .822; .041
Stroop – W&C [c]	3.12; 12.98	28.56; 13.10	$.660_{(144)}$; .511; .121
SRT – LTS	26.26; 13.73	22.7; 13.61	$1.444_{(147)}$; .151; .261
SRT – CLTR	16.04; 12.90	14.39; 12.91	$.713_{(147)}$; .477; .129
SRT – DR [d]	5.75; 2.85	5.39; 2.87	$.705_{(139)}$; .482; .129
SRT – Intrusions	2.70; 4.24	3.34; 3.49	$-.879_{(147)}$; .381; .159
CERAD – Total hits [e]	17.66; 4.79	17.35; 5.31	$.335_{(138)}$; .738; .063
CERAD – DR hits [e]	5.76; 2.47	5.70; 2.28	$.133_{(138)}$; .895; .025
MMSE	26.53; 3.45	26.52; 2.72	$.018_{(147)}$; .986; .003
MOCA [f]	17.95; 4.96	18.31; 5.57	$-.349_{(115)}$; .728; .071
Cognitive dimensions			$t_{(df)}$; *p*; Cohen's d
Executive [g]	.08; 1.04	-.21; .85	$.852_{(146)}$; .396; .291
Memory [h]	.05; .99	-.14; 1.02	$.754_{(146)}$; .452; .183

[a] n=138 [Iron sufficiency =98 (71.01%). ID=40 (28.99%)]; [b] n=143 [Iron sufficiency =102 (71.33%). ID=41 (28.67%)]; [c] n=146 [Iron sufficiency =103 (70.55%). ID=43 (29.45%)]; [d] n=141 [Iron sufficiency =97 (68.79%). ID=44 (31.21%)]; [e] n=140 [Iron sufficiency =100 (71.43%). ID=40 (28.57%)]; [f] n=117 [Iron sufficiency =82 (70.09%). ID=35 (29.91%)]; [g] n=141 [Iron sufficiency =100 (70.92%). ID=41 (29.08%)]; [h] n=132 [Iron sufficiency =92 (69.7%). ID=40 (30.3%)].

Table 13 - Functional ability and functional dimensions regarding iron status classification

Variables (mean; SD)	Iron sufficiency	ID	$t_{(df)}$; *p*; Cohen's d
Functional ability – QoFA [a]			$t_{(df)}$; *p*; Cohen's d
Mobility tiredness	4.55; 1.98	4.30; 1.76	$.732_{(146)}$; .465; .133
Lower limb tiredness	4.35; 1.15	4.02; 1.05	$1.608_{(146)}$; .110; .291
Upper limb tiredness	3.76; .81	3.82; .54	$-.441_{(146)}$; .660; .080
Mobility help	11.08; 1.99	11.14; 2.00	$-.166_{(146)}$; .868; .030
PADL help	18.87; 2.49	18.14; 3.27	$1.479_{(146)}$; .141; .268
Functional components [a]			$t_{(df)}$; *p*; Cohen's d
Functional tiredness	.06; 1.05	-.09; .75	$.852_{(146)}$; .396; .154
Functional help	.06; .92	-.07; 1.02	$.754_{(146)}$; .452; .137

[a] n=148 [Iron sufficiency =104 (70.27%); ID=44 (29.73%)].
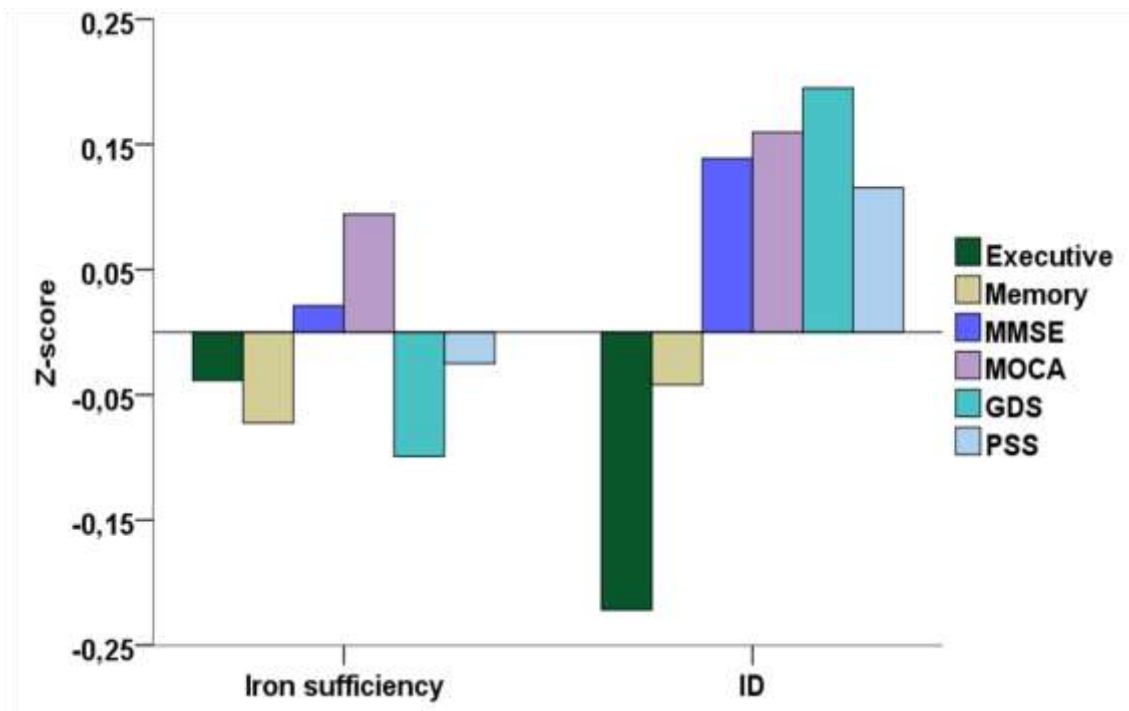
Figure 11 – Distribution of neurocognitive/neuropsychological dimensions by iron status groups.
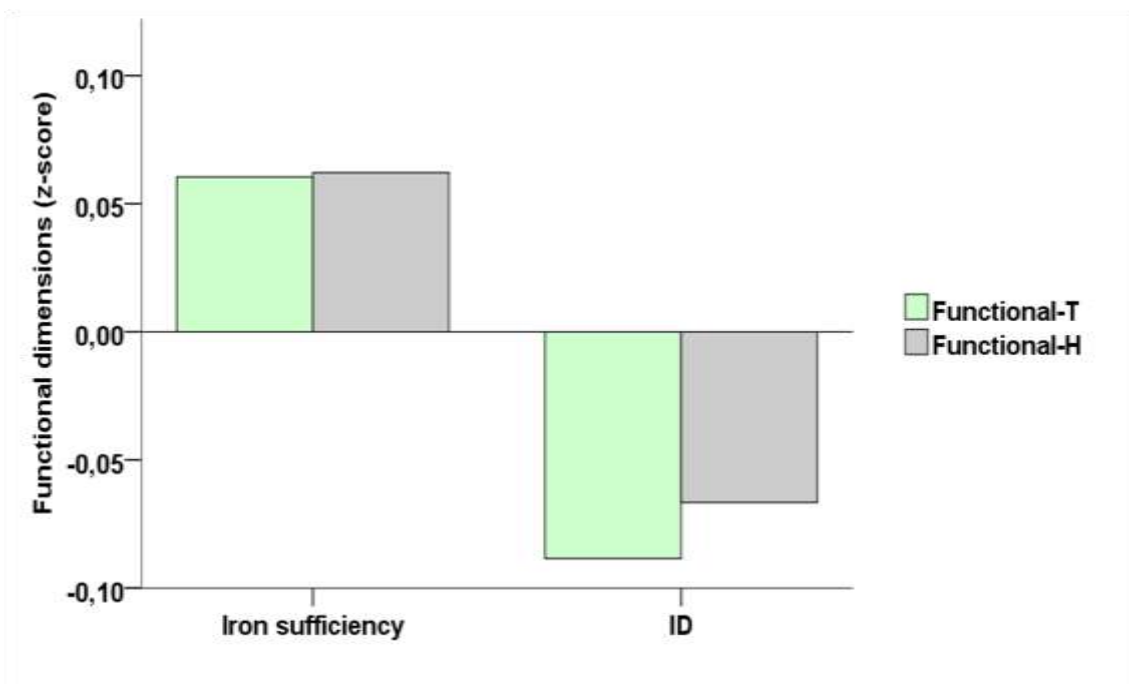
Figure 12 - Functional dimensions mean value by iron status groups.

Table 14 - Hematologic variables and dimensions in iron status groups.

Variables (mean; SD)	Iron sufficiency	ID	
Inflammatory indices			$Z_{(U)}$; ***p***; *r* [£]
hsCRP (mg/dL) [¥£]	1.56; 2.42	2.90; 2.27	$-2.880_{(1617.5)}$; .004; .236
Red cells indices [a]			$t_{(df)}$; ***p***; **Cohen's d**
RBC (10^{12}/L)	4.60; .43	4.45; .43	$1.911_{(144)}$; .058; .352
Hemoglobin (mg/dL)	14.17; 1.48	12.88; 1.56	$4.702_{(144)}$; <.001; .866
Hematocrit (%)	41.23; 3.83	38.23; 4.05	$4.205_{(144)}$; <.001; .774
MCV (fL)	89.61; 3.64	85.9; 4.78	$5.076_{(144)}$; <.001; .934
MCH (pg)	30.78; 1.44	28.92; 2.06	$5.354_{(57.8)}$; <.001; 1.144
MCHC (g/dL)	34.35; .92	33.65; .97	$4.075_{(144)}$; <.001; .750
RDW (%)	13.14; .64	13.95; 1.11	$-4.439_{(52.49)}$; <.001; 1.015
Iron biomarkers [b]			$t_{(df)}$; ***p***; **Cohen's d**
Fe (µg/dL)	102.83; 24.85	70.64; 27.12	$6.986_{(144)}$; <.001; 1.269
TF (mg/dL)	232.34; 28.96	285.43; 46.29	$-7.036_{(58.04)}$; <.001; 1.525
FT (mg/mL) [¥£]	173.00; 209.25	35.50; 41.25	$-7.190_{(557.5)}$; <.001; .595
TF sat. (%)	31.84; 8.06	18.87; 8.12	$8.906_{(144)}$; <.001; 1.617
TIBC (µg/dL)	326.30; 40,32	388.34; 61.23	$-6.168_{(59.7)}$; <.001; 1.314
sTFr (mg/L) [c¥£]	1.02; .34	1.475; .80	$-6.170_{(818)}$; <.001; .507
sTFr-LogFT index [¥£]	.483; .19	.888; .77	$-7.810_{(413)}$; <.001; .646
Body iron (mg/kg)	17.02; 2.66	10.02; 4.70	$9.249_{(55.2)}$; <.001; 2.072
Iron dimensions			$t_{(df)}$; ***p***; **Cohen's d**
Storage [b]	.43; .49	-1.01; 1.15	$7.997_{(49.73)}$; <.001; 1.936
Transport [b]	.37; .81	-.85; .87	$8.156_{(144)}$; <.001; 1.481
Transport S. [b]	.36; .70	-.84; 1.10	$6.666_{(58.55)}$; <.001; 1.437
Red cells C. [a]	.31; .72	-.77; 1.17	$5.575_{(54.05)}$; <.001; 1.247
Erythropoiesis [a]	.19; .95	-.46; .98	$3.707_{(144)}$; <.001; .682

[a] n=146 [Iron sufficiency =104 (71.23%). ID=42 (28.77%)]; [b] n=146 [Iron sufficiency =102 (69.86%). ID=44 (30.14%)]; [c] n=148 [Iron sufficiency =104 (70.27%). ID=44 (29.73%)]; [¥] Variables not normally distributed. data presented in median and interquartile range (median. IQR); [£] Mann–Whitney U test. results presented in $Z_{(U)}$; *p*; *r*.

As expected, significant differences were observed for almost all of the hematologic variables between groups of iron status (Table 14, Figure 13), with the iron sufficiency group with higher values. Of notice, hsCRP was significantly higher in the ID group, which can impact on the levels of iron biomarkers.
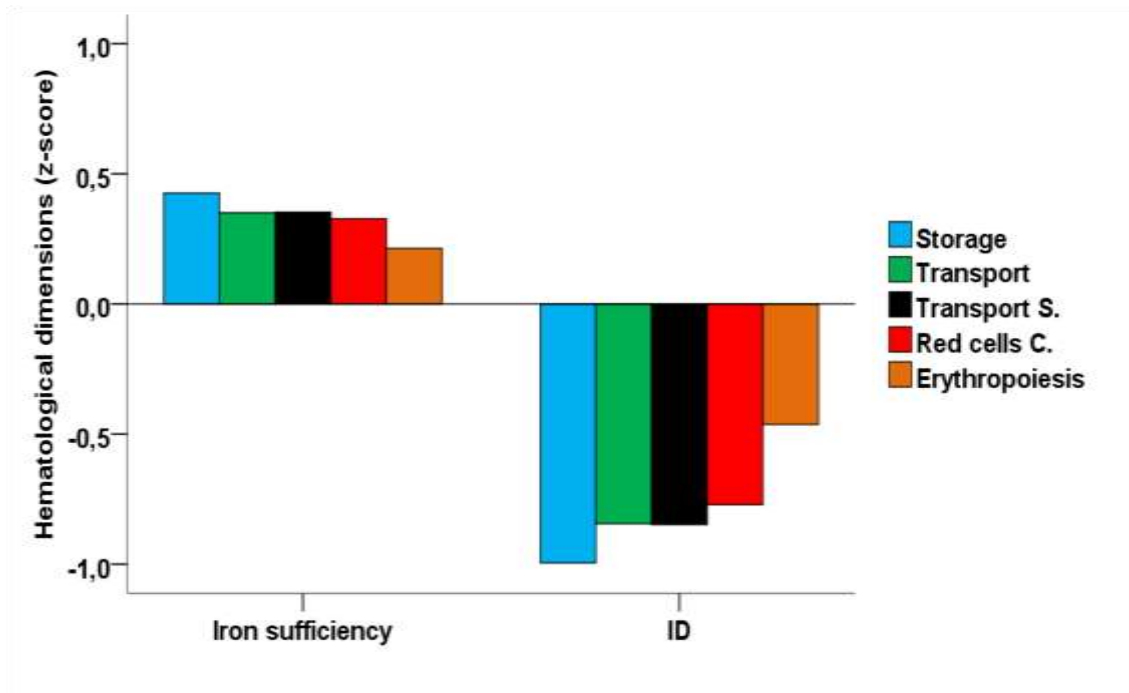
Figure 13 - Hematological dimensions by iron status groups.

Since several factors can contribute as confounding factors either for neurocognitive, neuropsychological or physical functional variables, ANCOVA was used to test differences in dependent variables (Executive, Memory, MMSE, MOCA, GDS, PSS, Functional-T and Functional-H) in iron status groups (iron sufficiency *versus* ID). Data presented in tables -15, -16, -17 and -18 indicate that there are no differences between groups of iron status when controlling for principal confounding factors, including for GDS which was significantly different before controlling.

Table 15 – ANCOVA for neurocognitive dimensions

Source	Executive $F_{(1;\,134)}$	p	$\eta^2_{partial}$	Memory $F_{(1;\,125)}$	p	$\eta^2_{partial}$
Age	20.674	<.001	.134	8.984	.003	.067
Education [a]	16.926	<.001	.112	6.453	.012	.049
Gender [b]	.969	.327	.007	1.562	.214	.012
hsCRP (Log)	.261	.610	.002	.008	.931	<.001
GDS	13.531	<.001	.092	21.386	<.001	.146
ID [c]	2.971	.087	.022	.781	.378	.006
R^2; $R^2_{adjusted}$	.398; .371			.296; .262		

[a] 4 years and less=0, more than 4 years=1; [b] female=0, male=1; [c] iron sufficiency=0, ID=1

Table 16 – ANCOVA for general cognition variables

	MMSE			MOCA		
Source	$F_{(1;\ 142)}$	*p*	η^2_{partial}	$F_{(1;\ 110)}$	*p*	η^2_{partial}
Age	12.773	<.001	.083	10.028	.002	.084
Education [a]	5.440	.021	.037	24.475	<.001	.182
Gender [b]	3.105	.080	.021	.892	.347	.008
hsCRP (Log)	.028	.867	<.001	.020	.889	<.001
GDS	9.868	.002	.065	9.235	.003	.077
ID [c]	.409	.523	.003	.014	.907	<.001
R^2; R^2_{adjusted}	.271; .240			.395; .362		

[a] 4 years and less=0, more than 4 years=1; [b] female=0, male=1; [c] iron sufficiency=0, ID=1

Table 17 - ANCOVA for neuropsychological variables

	GDS			PSS		
Source	$F_{(1;\ 149)}$	*p*	η^2_{partial}	$F_{(1;\ 138)}$	*p*	η^2_{partial}
Age	.003	.955	<.001	.040	.843	<.001
Education [a]	3.583	.060	.024	.952	.331	.007
Gender [b]	10.836	.001	.070	18.711	<.001	.124
hsCRP (Log)	.155	.695	.001	2.902	.091	.022
ID [c]	3.792	.053	.026	.085	.771	.001
R^2; R^2_{adjusted}	.148; .118			.138; .106		

[a] 4 years and less=0, more than 4 years=1; [b] female=0, male=1; [c] iron sufficiency=0, ID=1

Table 18 – ANCOVA for functional dimensions

	Functional-T			Functional-H		
Source	$F_{(1;\ 139)}$	*p*	η^2_{partial}	$F_{(1;\ 139)}$	*p*	η^2_{partial}
Age	4.228	.042	.030	4.718	.032	.034
BMI	8.251	.005	.058	16.179	<.001	.107
hsCRP (Log)	.020	.887	<.001	.852	.358	.006
ID [a]	.697	.405	.005	.418	.519	.003
R^2; R^2_{adjusted}	.116; .089			.153; .128		

[a] iron sufficiency=0, ID=1

4.1.3 Nutritional risk factors for iron deficiency

Dietary iron is the natural source of iron to the body; therefore, it is expected that ID is closely related to the individual's nutritional status. To test if nutritional status (MNA) and/or body composition (BMI, %BF-Tanita and %BF-Brozek) were predictors of ID, a logistic regression was conducted and controlled using the enter method for the potential confounding factors (age, gender and hsCRP). Results from the binary logistic regression are presented in Table 19. From

the variables introduced in model 1 (equal to MNA, BMI and %BF-Brozek adjusted models; different for %BF-Tanita due to missing values), as expected, we observed that gender ($Wald_{(1)}$= 6.926; p= .008) and hsCRP (Log) ($Wald_{(1)}$= 8.374; p= .004) were significant predictors of ID meaning that the odds of ID presence in males are 3.040 lower (OR = .329) than in females; and for each fold increase in hsCRP (Log) an increase of 5.278 (OR = 5.278) in the odds of the presence of ID is observed. After introducing nutritional status and body composition variables in the adjusted models 2 we observed slight differences in the previous mentioned control variables, but with the same tendency and significance as the previously mentioned. The Wald criterion demonstrated that nutritional status, measured by the MNA total score, made also a significant contribution to prediction of ID ($Wald_{(1)}$= 5.389; p= .020). For each unit increase (1 point in the MNA total score) in nutritional status the odds of ID presence decreased 1.211 times (OR = .826). Body composition variables did not contribute significantly to the prediction of ID.

Table 19 – Binary logistic regression of nutritional predictors for ID [a]

Independent variables	Model 1				Model 2			
	B	SE	Wald$_{(df)}$; p	OR (CI 95%)	B	SE	Wald$_{(df)}$; p	OR (CI 95%)
Age	-.033	.026	1.643$_{(1)}$; .200	.967 (.919; 1.018)	-.028	.027	1.141$_{(1)}$; .285	.972 (.923; 1.024)
Gender [b]	-1.113	.423	6.926$_{(1)}$; .008	.329 (.143; .753)	-.920	.435	4.478$_{(1)}$; .034	.398 (.170; .934)
hsCRP (Log)	1.663	.575	8.374$_{(1)}$; .004	5.278 (1.711; 16.284)	1.817	.598	9.236$_{(1)}$; .002	6.152 (1.906; 19.854)
MNA					-.191	.082	5.389$_{(1)}$; .020	.826 (.704; .971)
$\chi^2_{(df)}$; p; R^2 Nagelkerke	17.645$_{(3)}$; p=.001; .169				5.533$_{(1)}$; p=.019; .217			
Age	-.033	.026	1.643$_{(1)}$; .200	.967 (.919; 1.018)	-.031	.026	1.365$_{(1)}$; .243	.970 (.921; 1.021)
Gender [b]	-1.113	.423	6.926$_{(1)}$; .008	.329 (.143; .753)	-1.211	.435	7.746$_{(1)}$; .005	.298 (.127; .699)
hsCRP (Log)	1.663	.575	8.374$_{(1)}$; .004	5.278 (1.711; 16.284)	1.966	.634	9.625$_{(1)}$; .002	7.145 (2.063; 24.749)
BMI					-.074	.058	1.624$_{(1)}$; .203	.929 (.830; 1.040)
$\chi^2_{(df)}$; p; R^2 Nagelkerke	17.645$_{(3)}$; p=.001; .169				1.679$_{(1)}$; p=.195; .184			
Age	-.028	.026	1.141$_{(1)}$; .285	.972 (.924; 1.024)	-.026	.026	.940$_{(1)}$; .332	.975 (.926; 1.027)
Gender [b]	-1.103	.431	6.554$_{(1)}$; .010	.332 (.143; .772)	-1.625	.602	7.294$_{(1)}$; .007	.197 (.061; .640)
hsCRP (Log)	1.469	.577	6.478$_{(1)}$; .011	4.346 (1.402; 13.472)	1.742	.623	7.834$_{(1)}$; .005	5.711 (1.686; 19.347)
%BF-Tanita					-.048	.036	1.775$_{(1)}$; .183	.953 (.887; 1.023)
$\chi^2_{(df)}$; p; R^2 Nagelkerke	14.942$_{(3)}$; p=.002; .149				1.786$_{(1)}$; p=.181; .166			
Age	-.033	.026	1.643$_{(1)}$; .200	.967 (.919; 1.018)	-.024	.028	.730$_{(1)}$; .393	.976 (.924; 1.032)
Gender [b]	-1.113	.423	6.926$_{(1)}$; .008	.329 (.143; .753)	-1.740	.868	4.023$_{(1)}$; .045	.175 (.032; .961)
hsCRP (Log)	1.663	.575	8.374$_{(1)}$; .004	5.278 (1.711; 16.284)	1.825	.612	8.882$_{(1)}$; .003	6.203 (1.868; 20.602)
%BF-Brozek					-.053	.063	.713$_{(1)}$; .398	.948 (.839; 1.073)
$\chi^2_{(df)}$; p; R^2 Nagelkerke	17.645$_{(3)}$; p=.001; .169				.702$_{(1)}$; p=.402; .175			

[a] iron sufficiency=0, ID=1; [b] female=0, male=1;

4.1.4   Iron status as a possible predictor of cognition, mood and physical functional ability

Hierarchical regression models were tested with the major confounding factor and known predictors as independent variables to predict dependent variables (block 1: model 0; tables -18, -19, -20 and -21). Using the enter method, hematological dimensions were tested as predictors (independent variable) of target variables (dependent variables) (block 2: Storage – model 1; Transport – model 2; Transport S. – model 3; Red cells C. – model 4; Erythropoiesis – model 5).

Table 20 presents the results of hierarchical regression models for neurocognitive dimensions. Age, education (as a dummy variable), gender, hsCRP (log) and mood (GDS) were used in block 1, model 0 as independent variables, resulting in a significant predictor model for executive and memory dimensions. For the executive dimension, in block 1, model 0, age, education and mood were significant predictors and the model explained 37.1% ($R^2_{adjusted}$) of the variance. This pattern was verified also in Block 1 for all the remaining models, although no significant improvement in executive dimension variance explanation was obtained from block 2 (models 1 to 5), with the increment of executive dimension variance explanation near to 0% ($\Delta R^2_{adjusted}$).

For the memory dimension (Table 20, Figure 14), in block 1, model 0, age and mood were the sole significant predictors of memory dimension; however, the model with all the independent variables explained 26.9% ($R^2_{adjusted}$) of the memory dimension variance. The addition of hematological dimensions in block 2, resulted in a significant predictor model 1, where age, education, mood and the storage dimension were significant predictors and a significant predictor model 5, where erythropoiesis was a significant predictor and in addition to the control variables of model 1, gender was also seen as a significant predictor. The addition of storage dimension (block 2, model 1) resulted in a significant increment of 2% ($\Delta R^2_{adjusted}$) of the variance explanation when compared with model 0. Similar to model 1, the addition of erythropoiesis dimension (block 2, model 5) resulted in a significant increment of 2.6% ($\Delta R^2_{adjusted}$) of the memory dimension variance explanation when compared with model 0.

Table 20 - Hierarchical regression models to predict neurocognitive dimensions.

Model		Executive B (CI 95%)	SE	β	t; *p*	Memory B (CI 95%)	SE	β	t; *p*
0	Age	-.342 (-.481; -.204)	.070	-.351	-4.888; <.001	-.264 (-.418; -.109)	.078	-.270	-3.376; .001
	Education [a]	.533 (.209; .856)	.163	.238	3.261; .001	.343 (-.018; .703)	.182	.155	1.880; .063
	Gender [b]	.219 (-.063; .502)	.143	.112	1.534; .127	-.165 (-.478; .148)	.158	-.085	-1.045; .298
	hsCRP (Log)	.013 (-.118; .145)	.067	.014	.202; .840	-.001 (-.153; .151)	.077	-.001	-.011; .991
	GDS	-.282 (-.422; -.142)	.071	-.288	-3.994; <.001	-.388 (-.546; -.231)	.079	-.395	-4.893; <.001
R²adjusted; R²; ΔR²		.371; .394; .394				.269; .298; .298			
F(df1; df2) for ΔR²; *p*		F(5; 132)=17.132; <.001				F(5; 123)=10.437; <.001			
1	Age	-.329 (-.468; -.190)	.070	-.338	-4.692; <.001	-.241 (-.395; -.087)	.078	-.247	-3.096; .002
	Education [a]	.577 (.250; .903)	.165	.258	3.493; .001	.407 (.046; .768)	.182	.184	2.232; .027
	Gender [b]	.153 (-.141; .447)	.149	.078	1.028; .306	-.251 (-.570; .068)	.161	-.129	-1.559; .122
	hsCRP (Log)	.007 (-.124; .139)	.066	.007	.109; .914	.000 (-.150; .150)	.076	.000	-.001; .999
	GDS	-.265 (-.406; -.123)	.071	-.270	-3.709; <.001	-.369 (-.525; -.213)	.079	-.375	-4.689; <.001
	Storage	.109 (-.033; .250)	.071	.112	1.520; .131	.169 (.010; .329)	.080	.167	2.105; .037
R²adjusted; R²; ΔR²		.377; .404; .011				.289; .322; .025			
F(df1; df2) for ΔR²; *p*		F(1; 131)=2.31; .131				F(1; 122)=4.431; .037			
2	Age	-.343 (-.483; -.204)	.071	-.352	-4.866; <.001	-.273 (-.427; -.118)	.078	-.279	-3.497; .001
	Education [a]	.533 (.209; .857)	.164	.238	3.252; .001	.358 (-.001; .718)	.182	.162	1.972; .051
	Gender [b]	.213 (-.078; .504)	.147	.108	1.449; .150	-.218 (-.537; .101)	.161	-.112	-1.355; .178
	hsCRP (Log)	.017 (-.120; .153)	.069	.017	.239; .811	.032 (-.125; .189)	.079	.032	.406; .685
	GDS	-.280 (-.423; -.136)	.072	-.286	-3.862; <.001	-.368 (-.527; -.210)	.080	-.374	-4.595; <.001
	Transport	.013 (-.132; .158)	.073	.014	.181; .857	.124 (-.039; .287)	.082	.124	1.508; .134
R²adjusted; R²; ΔR²		.366; .394; .000				.277; .311; .013			
F(df1; df2) for ΔR²; *p*		F(1; 131)=.033; .857				F(1; 122)=2.273; .134			
3	Age	-.344 (-.483; -.205)	.070	-.353	-4.902; <.001	-.266 (-.420; -.111)	.078	-.272	-3.395; .001
	Education [a]	.523 (.197; .848)	.165	.234	3.178; .002	.320 (-.044; .685)	.184	.145	1.740; .084
	Gender [b]	.208 (-.078; .494)	.144	.106	1.441; .152	-.174 (-.488; .139)	.158	-.089	-1.100; .273
	hsCRP (Log)	.018 (-.115; .152)	.067	.019	.272; .786	.011 (-.143; .165)	.078	.011	.142; .887
	GDS	-.276 (-.418; -.134)	.072	-.282	-3.85; <.001	-.376 (-.536; -.216)	.081	-.382	-4.661; <.001
	Transport S.	.043 (-.102; .189)	.074	.042	.590; .556	.070 (-.089; .229)	.080	.070	.875; .383
R²adjusted; R²; ΔR²		.367; .395; .002				.268; .302; .004			
F(df1; df2) for ΔR²; *p*		F(1; 131)=.348; .556				F(1; 122)=.765; .383			
4	Age	-.313 (-.457; -.168)	.073	-.315	-4.272; <.001	-.233 (-.394; -.071)	.081	-.234	-2.856; .005
	Education [a]	.626 (.292; .960)	.169	.273	3.704; <.001	.453 (.081; .825)	.188	.200	2.413; .017
	Gender [b]	.149 (-.146; .443)	.149	.074	.997; .320	-.236 (-.559; .088)	.164	-.119	-1.440; .152
	hsCRP (Log)	.020 (-.115; .155)	.068	.021	.298; .767	.006 (-.149; .161)	.078	.006	.077; .938
	GDS	-.273 (-.416; -.129)	.073	-.271	-3.753; <.001	-.378 (-.539; -.218)	.081	-.375	-4.670; <.001
	Red cells C.	.093 (-.049; .234)	.072	.094	1.299; .196	.085 (-.077; .247)	.082	.084	1.040; .300
R²adjusted; R²; ΔR²		.364; .391; .008				.266; .300; .006			
F(df1; df2) for ΔR²; *p*		F(1; 131)=1.686; .196				F(1; 122)=1.082; .300			
5	Age	-.324 (-.469; -.179)	.073	-.327	-4.426; <.001	-.214 (-.373; -.056)	.080	-.216	-2.677; .008
	Education [a]	.604 (.266; .942)	.171	.264	3.535; .001	.523 (.154; .891)	.186	.231	2.804; .006
	Gender [b]	.187 (-.143; .517)	.167	.094	1.121; .264	-.416 (-.772; -.061)	.180	-.210	-2.317; .022
	hsCRP (Log)	.017 (-.119; .154)	.069	.018	.253; .801	-.008 (-.159; .143)	.077	-.008	-.105; .917
	GDS	-.281 (-.429; -.132)	.075	-.279	-3.738; <.001	-.336 (-.498; -.175)	.082	-.333	-4.119; <.001
	Erythropoiesis	.008 (-.165; .180)	.087	.007	.088; .930	.229 (.045; .412)	.093	.227	2.470; .015
R²adjusted; R²; ΔR²		.355; .384; .000				.295; .328; .034			
F(df1; df2) for ΔR²; *p*		F(1; 131)=.008; .930				F(1; 122)=6.102; .015			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1
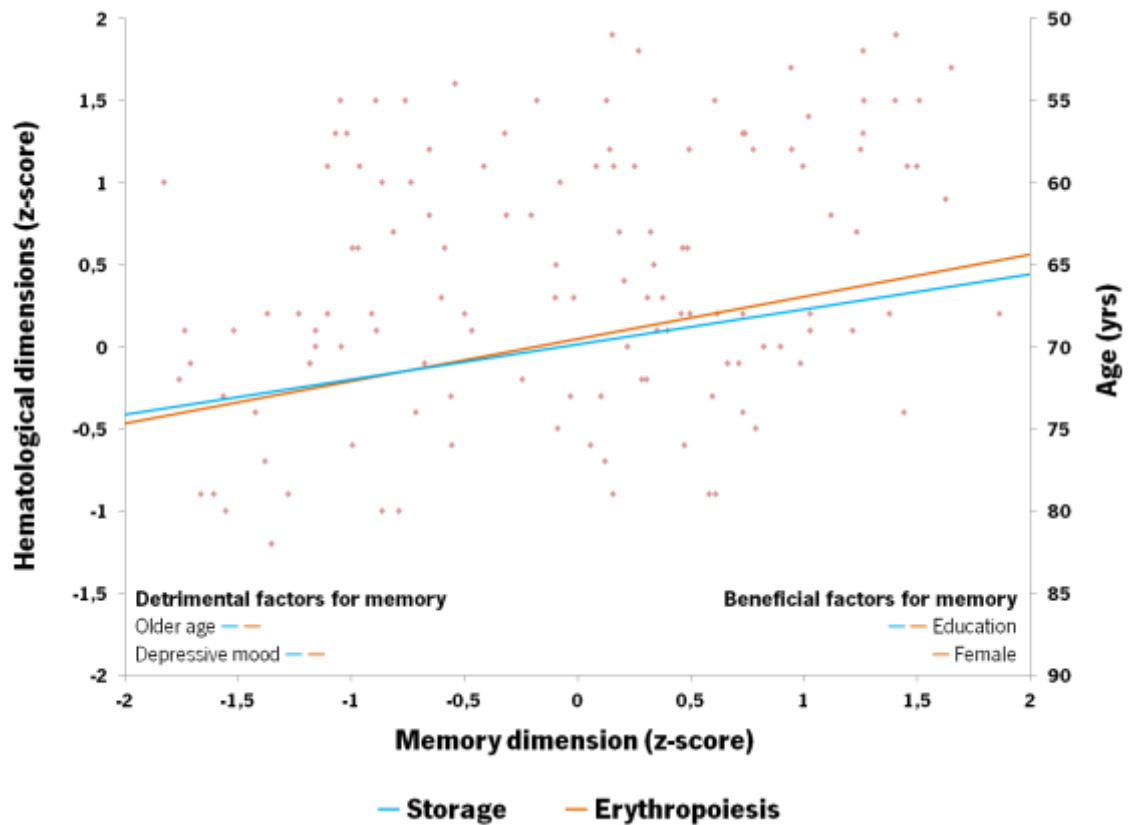
Figure 14 - Summary model for the correlation between memory dimension and hematological dimensions and associated beneficial and detrimental factors.

Hierarchical regression models to predict general cognition are presented in 21. Independent variables were the same as used in the hierarchical regression models for neurocognitive dimensions. The results obtained for general cognition were similar to those obtained for the executive dimension; block 1, model 0 (for MMSE and MOCA) had age, education and mood as significant predictors and the overall model explained 25.1% ($R^2_{adjusted}$) and 36.1% ($R^2_{adjusted}$) of MMSE and MOCA variance respectively. The pattern of significant predictors was maintained with the addition of hematological variables in block 2 (models 1 to 5), but hematological dimensions did not increment significant variance explanation for the MMSE or MOCA.

Table 21 - Hierarchical regression models to predict general cognition.

Model	MMSE B (CI 95%)	SE	β	t; p	MOCA B (CI 95%)	SE	β	t; p
0 Age	-.278 (-.419; -.136)	.072	-.295	-3.878; <.001	-.256 (-.415; -.098)	.080	-.255	-3.206; .002
Education [a]	.368 (.037; .698)	.167	.169	2.199; .030	.927 (.560; 1.294)	.185	.397	5.008; <.001
Gender [b]	.217 (-.073; .508)	.147	.115	1.478; .142	.133 (-.179; .446)	.158	.067	.847; .399
hsCRP (Log)	.036 (-.101; .173)	.069	.038	.524; .601	-.004 (-.154; .145)	.076	-.005	-.059; .953
GDS	-.239 (-.383; -.095)	.073	-.253	-3.284; .001	-.243 (-.397; -.089)	.078	-.243	-3.127; .002
$R^2_{adjusted}$; R^2; ΔR^2	.251; .277; .277				.365; .392; .392			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(5; 140)}$=10.719; <.001				$F_{(5; 109)}$=14.083; <.001			
1 Age	-.273 (-.415; -.130)	.072	-.290	-3.78; <.001	-.252 (-.411; -.093)	.080	-.251	-3.136; .002
Education [a]	.385 (.050; .720)	.170	.177	2.270; .025	.952 (.576; 1.328)	.190	.407	5.017; <.001
Gender [b]	.191 (-.111; .493)	.153	.101	1.250; .213	.113 (-.206; .433)	.161	.057	.704; .483
hsCRP (Log)	.033 (-.104; .171)	.069	.035	.481; .631	-.005 (-.155; .145)	.076	-.005	-.063; .950
GDS	-.232 (-.378; -.086)	.074	-.245	-3.146; .002	-.236 (-.392; -.081)	.079	-.236	-3.007; .003
Storage	.049 (-.096; .194)	.073	.051	.666; .506	.051 (-.109; .212)	.081	.050	.637; .525
$R^2_{adjusted}$; R^2; ΔR^2	.248; .279; .002				.361; .395; .002			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(1; 139)}$=.444; .506				$F_{(1; 108)}$=.406; .525			
2 Age	-.287 (-.429; -.145)	.072	-.305	-3.990; <.001	-.266 (-.426; -.105)	.081	-.265	-3.286; .001
Education [a]	.372 (.042; .702)	.167	.171	2.227; .028	.945 (.575; 1.315)	.187	.404	5.063; <.001
Gender [b]	.181 (-.115; .477)	.150	.095	1.208; .229	.101 (-.222; .423)	.163	.050	.619; .537
hsCRP (Log)	.057 (-.084; .197)	.071	.060	.801; .425	.013 (-.143; .169)	.079	.013	.164; .870
GDS	-.223 (-.369; -.077)	.074	-.236	-3.021; .003	-.230 (-.388; -.073)	.079	-.230	-2.907; .004
Transport	.091 (-.055; .238)	.074	.096	1.230; .221	.067 (-.094; .228)	.081	.069	.823; .413
$R^2_{adjusted}$; R^2; ΔR^2	.254; .285; .008				.363; .396; .004			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(1; 139)}$=1.514; .221				$F_{(1; 108)}$=.677; .413			
3 Age	-.279 (-.421; -.137)	.072	-.296	-3.882; <.001	-.258 (-.416; -.100)	.080	-.257	-3.238; .002
Education [a]	.358 (.025; .692)	.169	.165	2.124; .035	.903 (.536; 1.27)	.185	.386	4.876; <.001
Gender [b]	.211 (-.082; .504)	.148	.111	1.425; .156	.117 (-.195; .429)	.157	.059	.746; .457
hsCRP (Log)	.040 (-.098; .178)	.070	.043	.578; .564	.011 (-.140; .162)	.076	.011	.142; .888
GDS	-.232 (-.379; -.085)	.074	-.245	-3.123; .002	-.226 (-.381; -.070)	.078	-.226	-2.879; .005
Transport S.	.036 (-.106; .178)	.072	.038	.500; .618	.109 (-.050; .268)	.080	.105	1.359; .177
$R^2_{adjusted}$; R^2; ΔR^2	.247; .278; .001				.369; .403; .010			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(1; 139)}$=.250; .618				$F_{(1; 108)}$=1.847; .177			
4 Age	-.269 (-.420; -.118)	.076	-.275	-3.516; .001	-.255 (-.415; -.096)	.080	-.252	-3.181; .002
Education [a]	.429 (.081; .777)	.176	.189	2.435; .016	.935 (.561; 1.308)	.188	.395	4.961; <.001
Gender [b]	.213 (-.096; .522)	.156	.108	1.362; .175	.169 (-.151; .490)	.162	.084	1.047; .297
hsCRP (Log)	.033 (-.110; .176)	.072	.034	.455; .650	-.016 (-.167; .135)	.076	-.016	-.210; .834
GDS	-.231 (-.381; -.080)	.076	-.233	-3.032; .003	-.243 (-.398; -.088)	.078	-.240	-3.101; .002
Red cells C.	.050 (-.098; .199)	.075	.051	.670; .504	-.026 (-.183; .130)	.079	-.026	-.335; .739
$R^2_{adjusted}$; R^2; ΔR^2	.243; .274; .002				.363; .396; .001			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(1; 139)}$=.449; .504				$F_{(1; 110)}$=.112; .739			
5 Age	-.267 (-.418; -.116)	.076	-.273	-3.492; .001	-.235 (-.392; -.077)	.079	-.232	-2.957; .004
Education [a]	.439 (.089; .790)	.177	.193	2.480; .014	1.031 (.658; 1.404)	.188	.436	5.484; <.001
Gender [b]	.169 (-.173; .511)	.173	.086	.976; .331	-.011 (-.362; .341)	.177	-.005	-.060; .953
hsCRP (Log)	.029 (-.114; .171)	.072	.029	.399; .691	-.012 (-.160; .136)	.074	-.012	-.161; .872
GDS	-.218 (-.373; -.062)	.079	-.220	-2.766; .006	-.205 (-.362; -.049)	.079	-.203	-2.601; .011
Erythropoiesis	.073 (-.102; .247)	.088	.074	.822; .412	.175 (-.003; .353)	.090	.174	1.951; .054
$R^2_{adjusted}$; R^2; ΔR^2	.244; .276; .004				.383; .415; .020			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(1; 139)}$=.676; .412				$F_{(1; 110)}$=3.805; .054			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1

For neuropsychological variables the same independent variables were used as control variables and predictors, with exception of GDS which was, along with PSS, a dependent variable. Results from the hierarchical regression models for neuropsychological variables are presented in Table 22. Regarding GDS (Table 22, Figure 15) variance explanation for block 1 model 0, from all the independent variables, gender was the only significant predictor. Overall, model 0 was significant and explained 10.3% ($R^2_{adjusted}$) of GDS variance. The addition of hematological dimensions in block 2 resulted in the significant predictor models 2 (added transport), 3 (added transport S.) and 5 (added erythropoiesis). In block 2, model 2 the addition of storage component resulted in a significant increase in variance explanation of 2.1% ($\Delta R^2_{adjusted}$). Transport s. dimension addition (block 2, model 3) increased GDS variance explanation in 2.5% with statistical significant relevance. Block 2, model 5 was composed by the variables of model 0 and erythropoiesis dimension and resulted in a significant predictor model, which increased 4.1% ($\Delta R^2_{adjusted}$) the variance explanation when compared to Block 1 model 0. Of notice, gender is a significant predictor in all models with exception of model 5 where erythropoiesis is the only significant predictor with an impact that should be highlighted ($\beta = -.301$).

In block 1, model 0, results obtained for PSS were similar to the ones obtained for PSS; gender was the only significant predictor and the overall model explained 11.5% ($R^2_{adjusted}$) and 36.1% ($R^2_{adjusted}$) of PSS variance. Significance of gender as predictors was maintained with the addition of hematological variables in block 2 (models 1 to 5), but hematological dimensions did not increment significant variance explanation also for PSS.

Table 22 - Hierarchical regression models to predict neuropsychological dimensions.

Model		GDS B (CI 95%)	SE	β	t; p	PSS B (CI 95%)	SE	β	t; p
0	**Age**	-.009 (-.173; .154)	.083	-.009	-.113; .910	-.004 (-.175; .168)	.087	-.004	-.044; .965
	Education [a]	-.289 (-.668; .090)	.192	-.126	-1.505; .135	.216 (-.175; .608)	.198	.094	1.092; .277
	Gender [b]	-.628 (-.948; -.309)	.162	-.313	-3.888; <.001	-.745 (-1.079; -.41)	.169	-.369	-4.404; <.001
	hsCRP (Log)	.005 (-.153; .163)	.080	.005	.067; .947	-.155 (-.325; .015)	.086	-.151	-1.804; .074
	R²adjusted; R²; ΔR²	.103; .127; .127				.115; .141; .141			
	F(df1;df2) for ΔR²; p	F(4; 141)=5.143; .001				F(4; 130)=5.346; .001			
1	**Age**	-.024 (-.187; .140)	.083	-.024	-.289; .773	-.003 (-.176; .170)	.087	-.003	-.031; .976
	Education [a]	-.334 (-.714; .046)	.192	-.145	-1.735; .085	.220 (-.177; .618)	.201	.096	1.097; .275
	Gender [b]	-.537 (-.871; -.203)	.169	-.268	-3.179; .002	-.752 (-1.102; -.401)	.177	-.372	-4.244; <.001
	hsCRP (Log)	.013 (-.144; .171)	.080	.014	.170; .866	-.155 (-.326; .016)	.086	-.151	-1.798; .075
	Storage	-.144 (-.309; .020)	.083	-.144	-1.733; .085	.013 (-.167; .192)	.091	.012	.140; .889
	R²adjusted; R²; ΔR²	.115; .146; .018				.108; .141; 0			
	F(df1;df2) for ΔR²; p	F(1; 140)=3.002; .085				F(1; 129)=.020; .889			
2	**Age**	.008 (-.154; .171)	.082	.008	.101; .920	-.001 (-.175; .173)	.088	-.001	-.015; .988
	Education [a]	-.288 (-.662; .087)	.189	-.125	-1.519; .131	.215 (-.179; .608)	.199	.093	1.079; .283
	Gender [b]	-.538 (-.865; -.212)	.165	-.268	-3.257; .001	-.734 (-1.084; -.385)	.177	-.364	-4.161; <.001
	hsCRP (Log)	-.035 (-.196; .126)	.081	-.035	-.431; .667	-.161 (-.339; .018)	.090	-.156	-1.779; .078
	Transport	-.177 (-.342; -.012)	.084	-.176	-2.119; .036	-.019 (-.198; .160)	.091	-.019	-.209; .835
	R²adjusted; R²; ΔR²	.124; .154; .027				.108; .142; .000			
	F(df1;df2) for ΔR²; p	F(1; 140)=4.491; .036				F(1; 129)=.044; .835			
3	**Age**	-.003 (-.165; .158)	.082	-.003	-.039; .969	-.002 (-.174; .17)	.087	-.002	-.022; .982
	Education [a]	-.231 (-.609; .146)	.191	-.101	-1.213; .227	.245 (-.153; .643)	.201	.106	1.22; .225
	Gender [b]	-.574 (-.893; -.255)	.161	-.286	-3.561; .001	-.728 (-1.065; -.39)	.170	-.360	-4.269; <.001
	hsCRP (Log)	-.016 (-.173; .141)	.079	-.016	-.200; .842	-.166 (-.338; .006)	.087	-.162	-1.910; .058
	Transport S.	-.182 (-.341; -.023)	.080	-.181	-2.261; .025	-.075 (-.248; .098)	.088	-.072	-.855; .394
	R²adjusted; R²; ΔR²	.128; .158; .031				.113; .146; .005			
	F(df1;df2) for ΔR²; p	F(1; 140)=5.111; .025				F(1; 129)=.731; .394			
4	**Age**	.000 (-.167; .168)	.085	.000	.001; .999	-.037 (-.213; .139)	.089	-.036	-.413; .680
	Education [a]	-.259 (-.643; .125)	.194	-.113	-1.335; .184	.130 (-.270; .529)	.202	.056	.642; .522
	Gender [b]	-.550 (-.881; -.220)	.167	-.277	-3.291; .001	-.727 (-1.073; -.380)	.175	-.360	-4.151; <.001
	hsCRP (Log)	-.004 (-.162; .155)	.080	-.004	-.047; .962	-.157 (-.328; .014)	.087	-.153	-1.817; .072
	Red cells C.	-.077 (-.241; .087)	.083	-.078	-.929; .354	-.054 (-.235; .126)	.091	-.052	-.596; .552
	R²adjusted; R²; ΔR²	.085; .116; .005				.114; .147; .002			
	F(df1;df2) for ΔR²; p	F(1; 140)=.863; .354				F(1; 129)=.356; .552			
5	**Age**	-.026 (-.188; .136)	.082	-.026	-.318; .751	-.035 (-.212; .142)	.089	-.035	-.392; .696
	Education [a]	-.317 (-.689; .056)	.188	-.138	-1.681; .095	.131 (-.27; .533)	.203	.057	.647; .519
	Gender [b]	-.269 (-.634; .096)	.185	-.136	-1.458; .147	-.708 (-1.107; -.308)	.202	-.350	-3.505; .001
	hsCRP (Log)	.008 (-.145; .161)	.077	.008	.102; .919	-.152 (-.323; .019)	.086	-.148	-1.755; .082
	Erythropoiesis	-.298 (-.479; -.117)	.092	-.301	-3.26; .001	-.044 (-.247; .16)	.103	-.042	-.424; .672
	R²adjusted; R²; ΔR²	.144; .174; .063				.113; .146; .001			
	F(df1;df2) for ΔR²; p	F(1; 140)=10.627; .001				F(1; 129)=.180; .672			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1
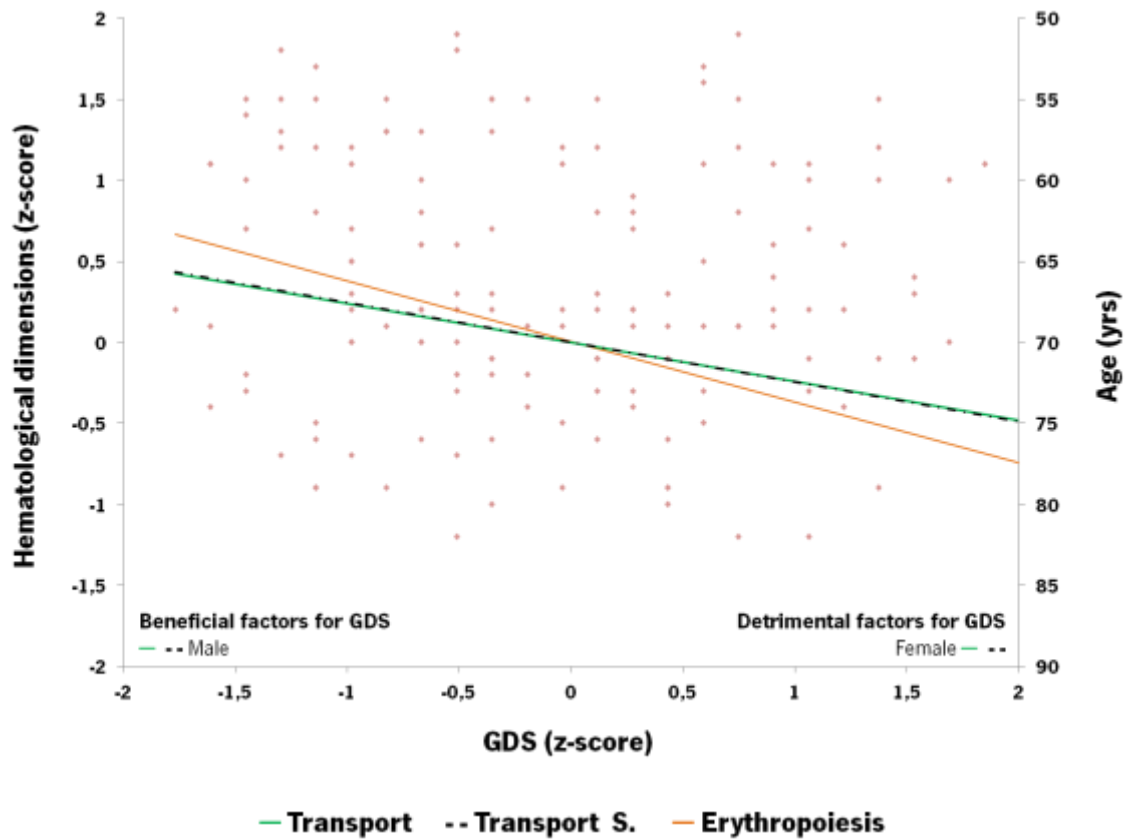
Figure 15 – Summary model for the correlation between depressive mood (GDS score) and hematological dimensions and associated beneficial and detrimental factors.

For the functional dimensions (Table 23), block 1 was composed by age, BMI and hsCRP (Log) as independent variables (control variables). In block 1, model 0, age and BMI were significant predictors and the overall model explained 8.8% ($R^2_{adjusted}$) and 13.9% ($R^2_{adjusted}$) of functional-T and functional-H variance respectively. Regarding functional-H dimension, the addition of hematological variables in block 2 (models 1 to 5) did not increment significant variance explanation; although, age and gender remained as significant predictors.

For the functional-T dimension (Table 23, Figure 16), after the addition of hematological dimensions in block 2 resulted in four models (models 1 - storage, 2 - transport, 4 – red cells c. and 5 – erythropoiesis) in which variance explanation was significantly increased. Transport S. addition in block 2 did not result in a significant increase in variance explanation. Storage, transport, red cells C. and erythropoiesis addition in block 2 resulted in a significant increase in variance explanation ($\Delta R^2_{adjusted}$) of 2.4%, 2.2%, 4.8% and 9.5% in model 1, 2 4 and 5 respectively.

Table 23 - Hierarchical regression models to predict functional dimensions.

Model		Functional-T				Functional-H			
		B (CI 95%)	SE	β	t; p	B (CI 95%)	SE	β	t; p
0	Age	-.165 (-.319; -.011)	.078	-.177	-2.116; .036	-.155 (-.298; -.012)	.072	-.175	-2.148; .033
	BMI	-.220 (-.387; -.054)	.084	-.231	-2.614; .010	-.315 (-.470; -.161)	.078	-.346	-4.033; <.001
	hsCRP (Log)	-.031 (-.198; .136)	.084	-.032	-.364; .716	.054 (-.101; .209)	.078	.058	.684; .495
$R^2_{adjusted}$; R^2; ΔR^2		.088; .108; .108				.139; .158; .158			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(3; 133)}$=5.353; .002				$F_{(3; 133)}$=8.344; <.001			
1	Age	-.151 (-.303; .002)	.077	-.162	-1.955; .053	-.151 (-.295; -.007)	.073	-.170	-2.078; .040
	BMI	-.234 (-.398; -.069)	.083	-.245	-2.805; .006	-.320 (-.475; -.164)	.079	-.350	-4.067; <.001
	hsCRP (Log)	-.020 (-.185; .145)	.083	-.021	-.241; .810	.057 (-.099; .213)	.079	.062	.723; .471
	Storage	.176 (.016; .336)	.081	.177	2.174; .032	.054 (-.097; .205)	.076	.057	.708; .480
$R^2_{adjusted}$; R^2; ΔR^2		.112; .139; .031				.136; .162; .003			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(1; 132)}$=4.725; .032				$F_{(1; 132)}$=.502; .480			
2	Age	-.179 (-.332; -.027)	.077	-.193	-2.323; .022	-.156 (-.300; -.012)	.073	-.175	-2.139; .034
	BMI	-.234 (-.399; -.069)	.083	-.246	-2.81; .006	-.316 (-.472; -.160)	.079	-.346	-4.011; <.001
	hsCRP (Log)	.026 (-.148; .199)	.088	.027	.293; .770	.056 (-.108; .219)	.083	.061	.673; .502
	Transport	.171 (.010; .333)	.082	.179	2.102; .037	.006 (-.146; .158)	.077	.007	.078; .938
$R^2_{adjusted}$; R^2; ΔR^2		.11; .137; .029				.133; .158; .000			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(1; 132)}$=4.419; .037				$F_{(1; 132)}$=.006; .938			
3	Age	-.165 (-.319; -.010)	.078	-.177	-2.107; .037	-.155 (-.299; -.012)	.073	-.175	-2.141; .034
	BMI	-.221 (-.388; -.053)	.084	-.231	-2.611; .010	-.315 (-.471; -.160)	.079	-.346	-4.017; <.001
	hsCRP (Log)	-.024 (-.194; .146)	.086	-.025	-.277; .782	.053 (-.105; .211)	.080	.058	.663; .509
	Transport S.	.038 (-.124; .200)	.082	.039	.461; .646	-.004 (-.154; .147)	.076	-.004	-.049; .961
$R^2_{adjusted}$; R^2; ΔR^2		.082; .109; .001				.133; .158; .000			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(1; 132)}$=.212; .646				$F_{(1; 132)}$=.002; .961			
4	Age	-.145 (-.298; .007)	.077	-.155	-1.883; .062	-.162 (-.308; -.017)	.074	-.182	-2.204; .029
	BMI	-.240 (-.402; -.078)	.082	-.253	-2.937; .004	-.308 (-.463; -.154)	.078	-.341	-3.943; <.001
	hsCRP (Log)	.002 (-.164; .168)	.084	.002	.025; .980	.069 (-.089; .228)	.080	.075	.865; .389
	Red cells C.	.218 (.057; .379)	.081	.218	2.676; .008	.030 (-.124; .184)	.078	.031	.385; .700
$R^2_{adjusted}$; R^2; ΔR^2		.136; .162; .045				.131; .156; .001			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(1; 132)}$=7.159; .008				$F_{(1; 132)}$=.149; .700			
5	Age	-.130 (-.279; .019)	.075	-.139	-1.730; .086	-.156 (-.301; -.010)	.074	-.174	-2.115; .036
	BMI	-.224 (-.381; -.067)	.079	-.236	-2.819; .006	-.305 (-.459; -.151)	.078	-.337	-3.919; <.001
	hsCRP (Log)	-.019 (-.179; .140)	.081	-.020	-.240; .811	.067 (-.089; .224)	.079	.073	.851; .396
	Erythropoiesis	.303 (.149; .458)	.078	.305	3.886; <.001	.076 (-.075; .227)	.076	.080	.997; .321
$R^2_{adjusted}$; R^2; ΔR^2		.183; .207; .091				.136; .162; .006			
$F_{(df1;df2)}$ for ΔR^2; **p**		$F_{(1; 132)}$=15.104; <.001				$F_{(1; 132)}$=.993; .321			
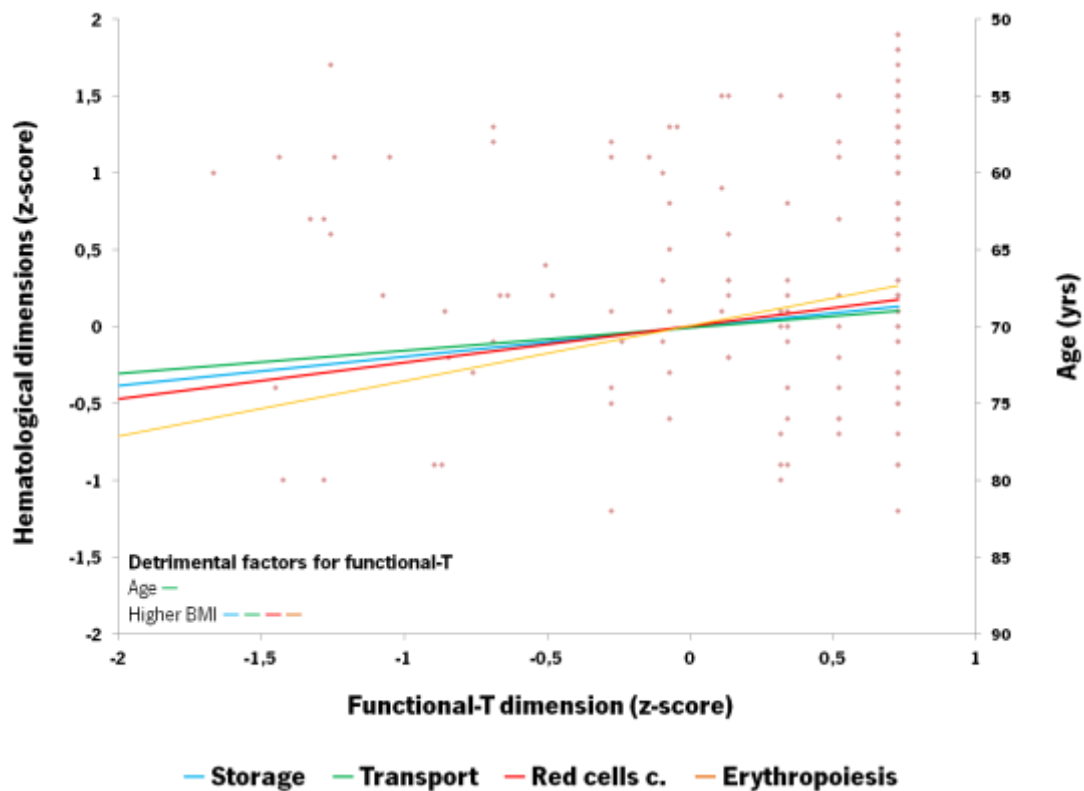
Figure 16 - Summary model for the correlation functional-T dimension and hematological dimensions and associated beneficial and detrimental factors.

4.1.5   Mediation effect of nutritional status on the iron status components

In order to assess if nutritional status (MNA) is a mediator of the hematological dimensions effect on the outcome of dependent variables (neurocognitive dimensions, general cognition, neuropsychological variables and functional dimensions) an interaction analysis on hierarchical multiple regression models was used. Variables carrying the information of the interaction between MNA and hematological dimensions were obtained by the product term of mean centered MNA score by the target hematological dimension (which is a z-score). Five new variables (MNA*Storage, MNA*Transport, MNA*Transport s., MNA*Red Cells c. and MNA*Erythropoiesis) were obtained and used, along with the origin variables, in the block 2 of similar hierarchical regression models previously presented.

Table 24 - Hierarchical regression models of interaction to predict neurocognitive dimensions.

Model	Executive B (CI 95%)	SE	β	t; p	Memory B (CI 95%)	SE	β	t; p
0 Age	-.327 (-.467; -.187)	.071	-.343	-4.631; <.001	-.263 (-.418; -.107)	.078	-.269	-3.345; .001
Education [a]	.484 (.156; .812)	.166	.220	2.922; .004	.346 (-.017; .709)	.183	.156	1.889; .061
Gender [b]	.232 (-.056; .520)	.146	.119	1.592; .114	-.160 (-.476; .155)	.159	-.082	-1.008; .315
hsCRP (Log)	.008 (-.129; .145)	.069	.008	.119; .905	-.002 (-.155; .150)	.077	-.002	-.030; .976
GDS	-.309 (-.454; -.163)	.074	-.311	-4.192; <.001	-.386 (-.545; -.228)	.080	-.393	-4.834; <.001
$R^2_{adjusted}$; R^2; ΔR^2	.366; .390; .390				.268; .297; .297			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(5;125)}$=16.005; <.001				$F_{(5;122)}$=10.313; <.001			
1 Age	-.327 (-.466; -.188)	.070	-.343	-4.649; <.001	-.245 (-.397; -.092)	.077	-.251	-3.182; .002
Education [a]	.510 (.177; .844)	.168	.232	3.03; .003	.369 (.012; .727)	.180	.167	2.048; .043
Gender [b]	.166 (-.131; .463)	.150	.085	1.106; .271	-.245 (-.560; .070)	.159	-.125	-1.542; .126
hsCRP (Log)	.004 (-.132; .140)	.069	.004	.055; .956	.013 (-.135; .161)	.075	.013	.176; .861
GDS	-.221 (-.383; -.060)	.081	-.223	-2.717; .008	-.331 (-.502; -.159)	.087	-.336	-3.82; <.001
MNA	.067 (.004; .131)	.032	.172	2.101; .038	.030 (-.037; .097)	.034	.076	.880; .381
Storage	.075 (-.078; .228)	.077	.074	.975; .332	.182 (.023; .340)	.080	.179	2.266; .025
MNA*Storage	.018 (-.028; .065)	.023	.056	.776; .439	.067 (.016; .117)	.025	.199	2.626; .010
$R^2_{adjusted}$; R^2; ΔR^2	.381; .419; .029				.317; .360; .063			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(3;122)}$=1.999; .118				$F_{(3;119)}$=3.907; .011			
2 Age	-.328 (-.468; -.188)	.071	-.344	-4.645; <.001	-.290 (-.445; -.135)	.078	-.297	-3.702; <.001
Education [a]	.492 (.166; .817)	.165	.223	2.989; .003	.327 (-.033; .687)	.182	.148	1.797; .075
Gender [b]	.226 (-.070; .523)	.150	.116	1.513; .133	-.234 (-.554; .086)	.162	-.120	-1.449; .150
hsCRP (Log)	-.018 (-.164; .127)	.073	-.019	-.247; .806	.067 (-.096; .229)	.082	.067	.815; .417
GDS	-.240 (-.402; -.078)	.082	-.241	-2.932; .004	-.340 (-.515; -.165)	.088	-.346	-3.853; <.001
MNA	.064 (.001; .126)	.032	.163	2.016; .046	.045 (-.031; .120)	.038	.113	1.175; .242
Transport	-.036 (-.187; .114)	.076	-.037	-.480; .632	.093 (-.075; .261)	.085	.093	1.096; .275
MNA*Transport	-.018 (-.075; .038)	.029	-.047	-.644; .521	.072 (-.001; .145)	.037	.169	1.956; .053
$R^2_{adjusted}$; R^2; ΔR^2	.377; .415; .025				.287; .332; .035			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(3;122)}$=1.723; .166				$F_{(3;119)}$=2.071; .108			
3 Age	-.337 (-.477; -.197)	.071	-.353	-4.763; <.001	-.259 (-.418; -.101)	.080	-.266	-3.242; .002
Education [a]	.502 (.162; .843)	.172	.228	2.922; .004	.304 (-.071; .679)	.189	.137	1.604; .111
Gender [b]	.198 (-.092; .488)	.146	.102	1.354; .178	-.183 (-.502; .136)	.161	-.094	-1.136; .258
hsCRP (Log)	.003 (-.136; .143)	.070	.004	.049; .961	.012 (-.145; .168)	.079	.012	.146; .884
GDS	-.232 (-.393; -.071)	.081	-.234	-2.85; .005	-.352 (-.531; -.173)	.090	-.358	-3.901; <.001
MNA	.064 (.001; .126)	.032	.163	2.027; .045	.025 (-.044; .094)	.035	.063	.717; .475
Transport A.	.019 (-.135; .172)	.078	.018	.240; .811	.060 (-.103; .224)	.082	.060	.731; .466
MNA*Transport S.	-.008 (-.067; .052)	.030	-.020	-.262; .794	.025 (-.041; .091)	.033	.060	.743; .459
$R^2_{adjusted}$; R^2; ΔR^2	.374; .413; .022				.26; .307; .009			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(3;122)}$=1.547; .206				$F_{(3;119)}$=.541; .655			
4 Age	-.315 (-.461; -.170)	.074	-.324	-4.286; <.001	-.238 (-.400; -.076)	.082	-.240	-2.911; .004
Education [a]	.571 (.233; .909)	.171	.253	3.34; .001	.425 (.052; .797)	.188	.188	2.258; .026
Gender [b]	.140 (-.162; .442)	.153	.071	.918; .361	-.264 (-.590; .062)	.165	-.133	-1.603; .112
hsCRP (Log)	.020 (-.123; .162)	.072	.020	.276; .783	.033 (-.126; .192)	.080	.033	.413; .680
GDS	-.227 (-.391; -.064)	.083	-.223	-2.751; .007	-.352 (-.530; -.175)	.090	-.349	-3.932; <.001
MNA	.074 (.010; .138)	.032	.187	2.292; .024	.036 (-.034; .106)	.036	.091	1.016; .312
Red Cells C.	.046 (-.114; .206)	.081	.044	.564; .574	.107 (-.063; .276)	.086	.105	1.247; .215
MNA*Red Cells C.	.019 (-.032; .070)	.026	.055	.731; .466	.049 (-.005; .104)	.028	.146	1.782; .077
$R^2_{adjusted}$; R^2; ΔR^2	.37; .409; .030				.275; .321; .027			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(3;122)}$=2.058; .109				$F_{(3;119)}$=1.58; .198			
5 Age	-.321 (-.467; -.175)	.074	-.330	-4.342; <.001	-.212 (-.373; -.051)	.081	-.214	-2.613; .010
Education [a]	.566 (.224; .908)	.173	.251	3.279; .001	.521 (.148; .895)	.189	.230	2.763; .007
Gender [b]	.164 (-.176; .504)	.172	.083	.955; .341	-.435 (-.794; -.076)	.181	-.219	-2.402; .018
hsCRP (Log)	.007 (-.134; .147)	.071	.007	.095; .924	-.001 (-.155; .153)	.078	-.001	-.015; .988
GDS	-.225 (-.392; -.058)	.084	-.220	-2.673; .009	-.296 (-.475; -.116)	.091	-.293	-3.264; .001
MNA	.073 (.009; .136)	.032	.183	2.271; .025	.034 (-.036; .104)	.035	.086	.964; .337
Erythropoiesis	.007 (-.175; .190)	.092	.007	.077; .939	.233 (.046; .420)	.094	.229	2.463; .015
MNA*Erythropoiesis	-.002 (-.055; .052)	.027	-.004	-.058; .954	.027 (-.031; .086)	.029	.075	.934; .352
$R^2_{adjusted}$; R^2; ΔR^2	.366; .405; .027				.293; .337; .044			
$F_{(df1;df2)}$ for ΔR^2; *p*	$F_{(3;122)}$=1.817; .148				$F_{(3;119)}$=2.618; .054			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1

An interaction effect was observed only in block 2, model 1 of the hierarchical regression model using memory dimension as focal dependent variable (Table 24, Figure 17). The overall model explained 31.7% ($R^2_{adjusted}$) of memory dimension variance. Age, education, mood, storage dimension and MNA*Storage were significant predictors, while gender, hsCRP (Log) and MNA were not. Interestingly, however, MNA score was a significant predictor of almost all dependent variables. MMSE and memory dimensions were the exceptions where MNA did not reveal predictive significance, regardless of being the only significant interaction observed in the memory dimension.
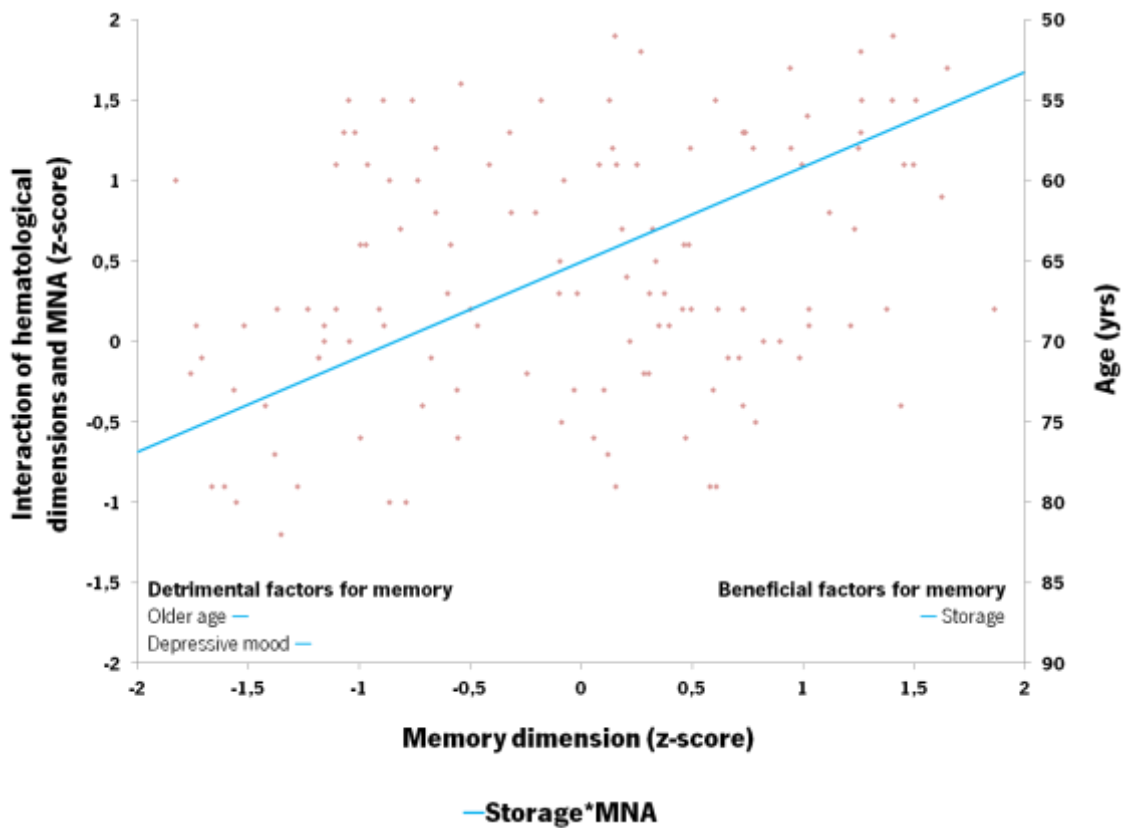

Figure 17 - Summary model for the correlation between memory dimension and interaction of storage dimension with nutritional status (MNA) and associated beneficial and detrimental factors.

Table 25 - Hierarchical regression models of interaction to predict general cognition

Model	MMSE				MOCA			
	B (CI 95%)	SE	β	t; p	B (CI 95%)	SE	β	t; p
0 Age	-.272 (-.416; -.128)	.073	-.293	-3.748; <.001	-.245 (-.400; -.089)	.078	-.247	-3.118; .002
Education [a]	.370 (.033; .707)	.170	.172	2.172; .032	.954 (.594; 1.314)	.182	.413	5.249; <.001
Gender [b]	.174 (-.124; .472)	.151	.092	1.154; .250	.165 (-.142; .473)	.155	.084	1.068; .288
hsCRP (Log)	.035 (-.108; .178)	.072	.036	.481; .631	-.015 (-.162; .132)	.074	-.015	-.198; .843
GDS	-.270 (-.420; -.120)	.076	-.281	-3.557; .001	-.229 (-.381; -.078)	.076	-.232	-3.004; .003
$R^2_{adjusted}$; R^2; ΔR^2	.255; .282; .282				.379; .406; .406			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(5; 131)}$=10.304; <.001				$F_{(5; 108)}$=14.775; <.001			
1 Age	-.275 (-.419; -.131)	.073	-.297	-3.787; <.001	-.252 (-.404; -.099)	.077	-.254	-3.274; .001
Education [a]	.362 (.020; .705)	.173	.168	2.091; .038	.958 (.597; 1.32)	.182	.415	5.26; <.001
Gender [b]	.143 (-.164; .450)	.155	.075	.920; .359	.139 (-.166; .445)	.154	.071	.904; .368
hsCRP (Log)	.036 (-.107; .179)	.072	.038	.499; .619	-.017 (-.161; .127)	.073	-.017	-.231; .818
GDS	-.204 (-.371; -.037)	.084	-.213	-2.421; .017	-.134 (-.297; .028)	.082	-.136	-1.642; .103
MNA	.052 (-.012; .116)	.032	.140	1.606; .111	.083 (.021; .146)	.032	.217	2.633; .010
Storage	.048 (-.108; .205)	.079	.048	.609; .544	.04 (-.115; .195)	.078	.040	.511; .611
MNA*Storage	.037 (-.012; .085)	.025	.114	1.484; .140	.033 (-.014; .080)	.024	.105	1.405; .163
$R^2_{adjusted}$; R^2; ΔR^2	.263; .306; .024				.409; .451; .045			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(3; 128)}$=1.465; .227				$F_{(3; 105)}$=2.845; .041			
2 Age	-.296 (-.440; -.153)	.073	-.32	-4.084; <.001	-.264 (-.418; -.109)	.078	-.266	-3.385; .001
Education [a]	.382 (.048; .715)	.169	.177	2.264; .025	.948 (.591; 1.304)	.180	.410	5.269; <.001
Gender [b]	.093 (-.211; .397)	.154	.049	.603; .548	.126 (-.185; .437)	.157	.064	.804; .423
hsCRP (Log)	.084 (-.068; .236)	.077	.088	1.097; .275	-.003 (-.156; .15)	.077	-.003	-.040; .968
GDS	-.204 (-.37; -.038)	.084	-.212	-2.432; .016	-.143 (-.305; .02)	.082	-.144	-1.74; .085
MNA	.048 (-.015; .111)	.032	.129	1.498; .137	.090 (.024; .156)	.033	.234	2.704; .008
Transport	.106 (-.048; .260)	.078	.111	1.365; .175	.006 (-.152; .165)	.080	.006	.077; .939
MNA*Transport	.044 (-.015; .103)	.030	.112	1.463; .146	.039 (-.024; .101)	.031	.097	1.227; .222
$R^2_{adjusted}$; R^2; ΔR^2	.271; .314; .032				.406; .448; .042			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(3; 128)}$=1.978; .120				$F_{(3; 105)}$=2.63; .054			
3 Age	-.270 (-.415; -.124)	.074	-.291	-3.666; <.001	-.246 (-.400; -.091)	.078	-.248	-3.147; .002
Education [a]	.342 (-.007; .690)	.176	.159	1.937; .055	.927 (.567; 1.288)	.182	.402	5.100; <.001
Gender [b]	.149 (-.152; .450)	.152	.079	.982; .328	.126 (-.177; .429)	.153	.064	.823; .412
hsCRP (Log)	.036 (-.11; .182)	.074	.038	.486; .628	-.010 (-.157; .136)	.074	-.010	-.137; .891
GDS	-.218 (-.386; -.049)	.085	-.226	-2.558; .012	-.140 (-.303; .023)	.082	-.142	-1.708; .091
MNA	.048 (-.015; .112)	.032	.130	1.51; .134	.078 (.016; .140)	.031	.202	2.496; .014
Transport A.	.008 (-.145; .161)	.077	.008	.102; .919	.061 (-.096; .218)	.079	.059	.773; .441
MNA*Transport S.	.028 (-.032; .088)	.030	.074	.930; .354	.025 (-.038; .088)	.032	.059	.776; .440
$R^2_{adjusted}$; R^2; ΔR^2	.254; .298; .015				.404; .446; .040			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(3; 128)}$=.938; .424				$F_{(3; 105)}$=2.532; .061			
4 Age	-.271 (-.424; -.118)	.077	-.281	-3.509; .001	-.258 (-.409; -.106)	.076	-.257	-3.377; .001
Education [a]	.425 (.071; .780)	.179	.189	2.373; .019	.935 (.580; 1.289)	.179	.400	5.231; <.001
Gender [b]	.135 (-.184; .453)	.161	.068	.837; .404	.176 (-.129; .482)	.154	.089	1.144; .255
hsCRP (Log)	.051 (-.101; .204)	.077	.052	.664; .508	-.015 (-.162; .131)	.074	-.016	-.209; .835
GDS	-.217 (-.389; -.044)	.087	-.215	-2.485; .014	-.152 (-.311; .008)	.080	-.152	-1.888; .062
MNA	.049 (-.016; .114)	.033	.127	1.482; .141	.091 (.029; .154)	.032	.235	2.889; .005
Red Cells C.	.064 (-.104; .231)	.085	.062	.750; .455	-.046 (-.202; .109)	.079	-.047	-.590; .557
MNA*Red Cells C.	.041 (-.013; .094)	.027	.119	1.511; .133	.042 (-.007; .090)	.025	.130	1.701; .092
$R^2_{adjusted}$; R^2; ΔR^2	.257; .301; .024				.420; .460; .051			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(3; 128)}$=1.471; .226				$F_{(3; 107)}$=3.376; .021			
5 Age	-.263 (-.417; -.109)	.078	-.272	-3.372; .001	-.232 (-.384; -.080)	.077	-.232	-3.028; .003
Education [a]	.464 (.104; .823)	.182	.206	2.552; .012	1.089 (.731; 1.447)	.181	.466	6.025; <.001
Gender [b]	.069 (-.287; .425)	.180	.035	.383; .702	-.021 (-.357; .316)	.170	-.010	-.121; .904
hsCRP (Log)	.028 (-.122; .178)	.076	.028	.370; .712	-.032 (-.174; .110)	.072	-.033	-.448; .655
GDS	-.189 (-.367; -.012)	.090	-.188	-2.113; .037	-.110 (-.271; .052)	.082	-.110	-1.342; .182
MNA	.048 (-.017; .114)	.033	.126	1.468; .145	.070 (.008; .132)	.031	.180	2.235; .027
Erythropoiesis	.106 (-.080; .292)	.094	.103	1.126; .262	.204 (.031; .376)	.087	.203	2.344; .021
MNA*Erythropoiesis	.015 (-.041; .071)	.028	.041	.535; .594	-.007 (-.059; .045)	.026	-.021	-.278; .782
$R^2_{adjusted}$; R^2; ΔR^2	.252; .296; .019				.429; .468; .059			
$F_{(df1;df2)}$ for ΔR^2; p	$F_{(3; 128)}$=1.155; .330				$F_{(3; 107)}$=3.959; .010			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1

Table 26 - Hierarchical regression models of interaction to predict neuropsychological variables.

Model		GDS B (CI 95%)	SE	β	t; p	PSS B (CI 95%)	SE	β	t; p
0	Age	-.022 (-.187; .142)	.083	-.023	-.268; .789	-.018 (-.184; .148)	.084	-.018	-.210; .834
	Education [a]	-.299 (-.682; .084)	.194	-.133	-1.544; .125	.187 (-.192; .566)	.192	.083	.975; .331
	Gender [b]	-.595 (-.921; -.268)	.165	-.301	-3.6; <.001	-.782 (-1.106; -.458)	.164	-.397	-4.778; <.001
	hsCRP (Log)	.004 (-.160; .169)	.083	.004	.050; .960	-.141 (-.305; .024)	.083	-.141	-1.694; .093
$R^2_{adjusted}$; R^2; ΔR^2		.094; .121; .121				.133; .160; .160			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(4, 132)}$=4.54; .002				$F_{(4, 129)}$=6.121; <.001			
1	Age	.000 (-.150; .150)	.076	.000	-.006; .995	.021 (-.140; .181)	.081	.021	.253; .801
	Education [a]	-.276 (-.630; .078)	.179	-.123	-1.541; .126	.271 (-.099; .641)	.187	.121	1.449; .150
	Gender [b]	-.376 (-.689; -.063)	.158	-.190	-2.375; .019	-.703 (-1.029; -.377)	.165	-.357	-4.264; <.001
	hsCRP (Log)	.015 (-.134; .164)	.075	.015	.200; .842	-.144 (-.302; .014)	.08	-.144	-1.806; .073
	MNA	-.165 (-.225; -.105)	.031	-.427	-5.409; <.001	-.126 (-.192; -.061)	.033	-.313	-3.839; <.001
	Storage	-.070 (-.233; .093)	.082	-.068	-.851; .397	.057 (-.111; .226)	.085	.056	.674; .502
	MNA*Storage	-.026 (-.077; .024)	.026	-.079	-1.024; .308	-.025 (-.079; .029)	.027	-.071	-.903; .368
$R^2_{adjusted}$; R^2; ΔR^2		.260; .298; .177				.206; .248; .089			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(3, 129)}$=10.863; <.001				$F_{(3, 126)}$=4.948; .003			
2	Age	.017 (-.134; .167)	.076	.017	.217; .829	.012 (-.149; .173)	.081	.012	.149; .882
	Education [a]	-.281 (-.628; .066)	.175	-.125	-1.603; .111	.264 (-.100; .629)	.184	.118	1.436; .153
	Gender [b]	-.359 (-.672; -.046)	.158	-.182	-2.272; .025	-.697 (-1.022; -.372)	.164	-.354	-4.247; <.001
	hsCRP (Log)	-.012 (-.172; .147)	.081	-.012	-.152; .879	-.137 (-.305; .031)	.085	-.137	-1.610; .110
	MNA	-.159 (-.219; -.099)	.030	-.411	-5.218; <.001	-.138 (-.207; -.070)	.035	-.343	-4.007; <.001
	Transport	-.094 (-.255; .067)	.081	-.095	-1.157; .250	.086 (-.084; .256)	.086	.087	1.002; .319
	MNA*Transport	-.009 (-.071; .053)	.031	-.022	-.283; .778	-.045 (-.115; .025)	.035	-.107	-1.279; .203
$R^2_{adjusted}$; R^2; ΔR^2		.259; .297; .176				.213; .255; .095			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(3, 129)}$=10.765; <.001				$F_{(3, 126)}$=5.356; .002			
3	Age	.011 (-.140; .161)	.076	.011	.142; .887	.014 (-.148; .175)	.081	.014	.168; .867
	Education [a]	-.242 (-.600; .117)	.181	-.108	-1.333; .185	.219 (-.158; .595)	.190	.097	1.147; .254
	Gender [b]	-.383 (-.687; -.078)	.154	-.194	-2.488; .014	-.676 (-.995; -.357)	.161	-.343	-4.193; <.001
	hsCRP (Log)	-.001 (-.153; .150)	.077	-.001	-.014; .989	-.134 (-.295; .028)	.082	-.134	-1.641; .103
	MNA	-.156 (-.216; -.097)	.030	-.405	-5.183; <.001	-.117 (-.183; -.052)	.033	-.290	-3.552; .001
	Transport A.	-.111 (-.268; .046)	.079	-.109	-1.404; .163	.015 (-.151; .181)	.084	.015	.182; .856
	MNA*Transport S.	.002 (-.060; .064)	.031	.005	.068; .946	.007 (-.063; .078)	.036	.017	.205; .838
$R^2_{adjusted}$; R^2; ΔR^2		.262; .3; .179				.198; .241; .081			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(3, 129)}$=10.995; <.001				$F_{(3, 126)}$=4.48; .005			
4	Age	.029 (-.125; .184)	.078	.031	.378; .706	-.006 (-.171; .159)	.083	-.006	-.075; .941
	Education [a]	-.231 (-.588; .125)	.180	-.104	-1.286; .201	.166 (-.206; .538)	.188	.073	.882; .380
	Gender [b]	-.385 (-.700; -.071)	.159	-.197	-2.426; .017	-.707 (-1.031; -.383)	.164	-.359	-4.314; <.001
	hsCRP (Log)	.013 (-.141; .167)	.078	.013	.164; .870	-.128 (-.290; .035)	.082	-.128	-1.556; .122
	MNA	-.158 (-.218; -.098)	.030	-.414	-5.218; <.001	-.118 (-.184; -.051)	.034	-.293	-3.515; .001
	Red Cells C.	.006 (-.163; .176)	.086	.006	.073; .942	.043 (-.132; .218)	.089	.042	.484; .629
	MNA*Red Cells C.	.008 (-.046; .061)	.027	.023	.283; .777	.015 (-.041; .072)	.029	.045	.540; .590
$R^2_{adjusted}$; R^2; ΔR^2		.230; .269; .165				.208; .250; .085			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(3, 129)}$=9.725; <.001				$F_{(3, 126)}$=4.785; .003			
5	Age	-.002 (-.154; .149)	.077	-.002	-.029; .977	-.013 (-.179; .152)	.084	-.013	-.157; .875
	Education [a]	-.297 (-.646; .053)	.177	-.133	-1.68; .095	.148 (-.228; .524)	.190	.066	.780; .437
	Gender [b]	-.144 (-.492; .205)	.176	-.074	-.815; .417	-.663 (-1.033; -.292)	.187	-.336	-3.539; .001
	hsCRP (Log)	.006 (-.142; .154)	.075	.006	.081; .936	-.136 (-.296; .024)	.081	-.136	-1.686; .094
	MNA	-.155 (-.213; -.097)	.029	-.406	-5.264; <.001	-.115 (-.181; -.048)	.034	-.285	-3.422; .001
	Erythropoiesis	-.226 (-.404; -.047)	.090	-.222	-2.503; .014	-.026 (-.219; .166)	.097	-.026	-.268; .789
	MNA*Erythropoiesis	-.019 (-.074; .036)	.028	-.053	-.690; .492	.008 (-.052; .068)	.030	.023	.275; .784
$R^2_{adjusted}$; R^2; ΔR^2		.267; .305; .201				.206; .248; .084			
$F_{(df1;df2)}$ for ΔR^2; p		$F_{(3, 129)}$=12.399; <.001				$F_{(3, 126)}$=4.677; .004			

[a] Less than 4 school years=0, More than 4 school years=1 ; [b] Female=0, Male=1

Table 27 - Hierarchical regression models of interaction to predict functional dimensions.

Model	Functional-T B (CI 95%)	SE	β	t; p	Functional-H B (CI 95%)	SE	β	t; p
0 Age	-.165 (-.319; -.011)	.078	-.177	-2.116; .036	-.155 (-.298; -.012)	.072	-.175	-2.148; .033
BMI	-.220 (-.387; -.054)	.084	-.231	-2.614; .010	-.315 (-.47; -.161)	.078	-.346	-4.033; <.001
hsCRP (Log)	-.031 (-.198; .136)	.084	-.032	-.364; .716	.054 (-.101; .209)	.078	.058	.684; .495
R²adjusted; R²; ΔR²	.088; .108; .108				.139; .158; .158			
F(df1;df2) for ΔR²; p	F(3; 133)=5.353; .002				F(3; 133)=8.344; <.001			
1 Age	-.173 (-.318; -.027)	.073	-.186	-2.350; .020	-.163 (-.305; -.022)	.071	-.184	-2.287; .024
BMI	-.196 (-.356; -.036)	.081	-.206	-2.421; .017	-.279 (-.435; -.123)	.079	-.306	-3.546; .001
hsCRP (Log)	-.043 (-.200; .115)	.080	-.044	-.535; .594	.041 (-.112; .195)	.077	.045	.536; .593
MNA	.116 (.056; .176)	.030	.311	3.803; <.001	.079 (.020; .137)	.030	.220	2.652; .009
Storage	.096 (-.062; .254)	.080	.096	1.203; .231	.015 (-.139; .168)	.078	.015	.187; .852
MNA*Storage	-.010 (-.061; .041)	.026	-.030	-.370; .712	.029 (-.02; .079)	.025	.095	1.162; .247
R²adjusted; R²; ΔR²	.197; .233; .125				.17; .207; .048			
F(df1;df2) for ΔR²; p	F(3; 130)=7.050; <.001				F(3; 130)=2.641; .052			
2 Age	-.186 (-.332; -.041)	.073	-.201	-2.538; .012	-.167 (-.308; -.025)	.071	-.187	-2.333; .021
BMI	-.195 (-.354; -.036)	.080	-.205	-2.43; .016	-.279 (-.434; -.125)	.078	-.306	-3.576; <.001
hsCRP (Log)	-.024 (-.193; .144)	.085	-.025	-.287; .775	.044 (-.120; .207)	.083	.048	.529; .598
MNA	.116 (.056; .176)	.030	.311	3.829; <.001	.082 (.024; .140)	.029	.231	2.797; .006
Transport	.096 (-.061; .254)	.080	.101	1.209; .229	-.044 (-.198; .109)	.078	-.049	-.574; .567
MNA*Transport	-.015 (-.077; .047)	.032	-.038	-.476; .635	.035 (-.025; .096)	.031	.094	1.148; .253
R²adjusted; R²; ΔR²	.197; .233; .125				.172; .208; .050			
F(df1;df2) for ΔR²; p	F(3; 130)=7.049; <.001				F(3; 130)=2.731; .046			
3 Age	-.186 (-.334; -.038)	.075	-.200	-2.488; .014	-.146 (-.288; -.003)	.072	-.164	-2.025; .045
BMI	-.184 (-.344; -.024)	.081	-.193	-2.269; .025	-.272 (-.426; -.117)	.078	-.298	-3.48; .001
hsCRP (Log)	-.055 (-.216; .106)	.081	-.057	-.676; .500	.031 (-.124; .185)	.078	.033	.393; .695
MNA	.127 (.068; .187)	.030	.342	4.228; <.001	.084 (.026; .141)	.029	.235	2.887; .005
Transport A.	-.025 (-.181; .130)	.079	-.026	-.319; .750	-.051 (-.201; .099)	.076	-.054	-.672; .503
MNA*Transport S.	-.010 (-.072; .052)	.031	-.025	-.307; .759	.047 (-.013; .106)	.030	.127	1.548; .124
R²adjusted; R²; ΔR²	.188; .224; .116				.178; .215; .056			
F(df1;df2) for ΔR²; p	F(3; 130)=6.495; <.001				F(3; 130)=3.109; .029			
4 Age	-.168 (-.314; -.022)	.074	-.179	-2.278; .024	-.173 (-.317; -.029)	.073	-.193	-2.382; .019
BMI	-.199 (-.355; -.044)	.079	-.210	-2.534; .012	-.284 (-.437; -.131)	.077	-.314	-3.674; <.001
hsCRP (Log)	-.018 (-.180; .143)	.082	-.019	-.225; .822	.072 (-.087; .231)	.080	.078	.895; .372
MNA	.116 (.057; .175)	.030	.310	3.919; <.001	.074 (.017; .132)	.029	.209	2.553; .012
Red Cells C.	.154 (-.008; .316)	.082	.154	1.876; .063	.010 (-.150; .169)	.081	.010	.119; .905
MNA*Red Cells C.	.010 (-.043; .063)	.027	.031	.383; .702	.032 (-.020; .084)	.026	.102	1.232; .220
R²adjusted; R²; ΔR²	.216; .250; .134				.165; .202; .047			
F(df1;df2) for ΔR²; p	F(3; 130)=7.749; <.001				F(3; 130)=2.529; .060			
5 Age	-.152 (-.296; -.009)	.073	-.162	-2.100; .038	-.162 (-.307; -.017)	.073	-.181	-2.211; .029
BMI	-.189 (-.340; -.037)	.077	-.199	-2.465; .015	-.281 (-.434; -.128)	.077	-.310	-3.631; <.001
hsCRP (Log)	-.036 (-.189; .118)	.078	-.037	-.462; .645	.064 (-.091; .219)	.078	.070	.819; .414
MNA	.109 (.052; .167)	.029	.293	3.785; <.001	.071 (.013; .129)	.029	.199	2.428; .017
Erythropoiesis	.245 (.095; .396)	.076	.247	3.224; .002	.040 (-.112; .191)	.077	.042	.515; .608
MNA*Erythropoiesis	.003 (-.051; .057)	.027	.008	.099; .922	.027 (-.028; .081)	.028	.079	.973; .333
R²adjusted; R²; ΔR²	.254; .287; .171				.163; .200; .045			
F(df1;df2) for ΔR²; p	F(3; 130)=10.389; <.001				F(3; 130)=2.418; .069			

4.2. Longitudinal analysis: iron fortification, cognition, mood and physical performance

From the n=151 individuals assessed in Moment A1, n=41 were included in the quasi-experimental intervention study after meeting inclusion (presence of ID) and exclusion criteria, as previously described, and accepting to continue in the study. Of these, n=1 individual was excluded due to initiation of intravenous iron treatment, and n=18 dropped-out (45%). From the dropouts two considerations should be addressed, firstly the dropouts were older than the ones that continued in the intervention and, secondly, dropouts had higher need of help to mobility (lower score on mobility help of physical functional ability) (data not shown) and these two factors may possibly explain the dropout of such higher number of individuals. The study sample for the study was, therefore, of 22 individuals, of which 10 (43%) were allocated to a non-intervention branch and 12 (57%) to a fortification branch. Allocation of participants had in consideration a similar distribution of individuals with lower levels of serum FT [FT<45 (µg/L)].

4.2.1 Characterization of participants

Socio-demographic and anthropometric characterization of participants is presented in Table 28. No significant differences were observed for all variables. Effect size (Cohen's d) was very small and perhaps our sample size does not have enough statistical power to detect differences of this magnitude.

Regarding neuropsychological assessment (Table 29), no statistical significant differences were observed between the groups for mood (measured by GDS) or perceived stress (assessed with the PSS). Of notice the absence of differences may be due to the small sample size as can be concluded by the small Cohen's d (<.2). In neurocognitive variables and dimensions from the cross sectional analyses (Table 29), no differences were observed between the supplemented and non supplemented groups.

Table 28 - Characteristics comparison for participants.

Variables (mean; SD)	Non supplem. (n=10)	Supplem. (n=12)	
Socio-demographic			t(df); *p*; Cohen's d
Age (years)	62.30; 4.64	64.58; 6.60	-.949(19.530); .354; .399
Education (school yrs)	6.50; 3.92	6.08; 3.80	.252(20); .803; .119
Anthropometric			t(df); *p*; Cohen's d
Weight (kg)	69.87; 8.08	75.58; 11.15	-1.350(20); .192; .586
Height (m)	1.60; .07	1.62; .09	-.606(20); .551; .248
BMI (kg/m^2)	27.43; 3.44	29.06; 5.2	-.848(20); .407; .369
Waist circ. (cm)	91.47; 10.51	93.07; 13.14	-.309(20); .760; .134
Hip circ. (cm)	101.18; 6.90	98.44; 14.63	.542(20); .594; .239
%BF-BIA (%)[a]	31.80; 9.31	33.75; 12.03	-.405(18); .690; .181
%BF-Brozek (%)[a]	34.06; 8.32	36.11; 8.12	-.583(20); .567; .249
Gender (n; %)			*p*(Fisher exact test; 2 tailed)
Females	6; 27.27	7; 31.81	>.999
Males	4; 18.18	5; 27.72	
BMI class (n; %)			*p*(Fisher exact test; 2 tailed)
Normal	2; 9.09	3; 13.63	.554
Overweight	6; 27.27	4; 18.18	
Obesity	2; 9.09	5; 22.72	
Nutritional status (n; %)			*p*(Fisher exact test; 2 tailed)
Risk of malnutrition	4; 18.18	2; 9.09	.348
Normal	6; 27.27	10; 45.45	

[a] n=20 [Non supplemented=10 (50%), Supplemented=10 (50%)].

Table 30 lists data regarding physical functional ability at the baseline, values for the functional dimensions were the same derived from the cross-sectional analysis. No significant differences were observed for all the variables with exception for lower limb tiredness ($t_{(14.228)}$=2.272; *p*= .039; d= .942) where the non supplemented group presented a higher mean score (meaning lower tiredness) than the supplemented group.

The characterization of groups and the comparison of means for hematological variables are presented in Table 31. No significant differences were observed for the inflammatory status. As expected, the distribution of individuals with low FT levels was similar between non supplemented and supplemented. Regarding the red cells indices, statistical differences were observed in MCV and MHC. In these two variables the mean value of the non supplemented group was higher than that of the supplemented group, indicating higher iron levels.

In iron biomarkers, Fe was significantly higher in the non supplemented group, the same pattern was observed for TF sat. and body iron. Statistical significant higher mean (or median)

values were observed for the supplemented group in TF, TIBC, sTFr and sTFr-LogFT index. In iron dimensions (values from the cross sectional anlalysis), erythropoiesis was not significantly different between groups; however, differences were observed in storage, transport, transport s. and red cells c In all of the significantly different dimensions the non supplemented group presented a higher mean value.

Table 29 - Neuropsychological and neurocognitive variables of participants.

Variables (mean, SD)	Non supplem.	Supplem.	
Psychological			$t_{(df)}$; *p*; Cohen's d
GDS	11.40; 5.74	11.33; 6.14	$.026_{(20)}$; .979; .012
PSS	17.70; 7.66	18.58; 6.32	$-.297_{(20)}$; .770; .125
Neurocognitive			$t_{(df)}$; *p*; Cohen's d
DS – Forward	6.20; 1.40	7.08; 1.93	$-1.206_{(20)}$; .242; .523
DS – Backward	4.10; 1.73	4.33; 1.44	$-.346_{(20)}$; .733; .144
DS – Total	10.30; 2.50	11.42; 2.94	$-9.49_{(20)}$; .354; .411
Stroop – W	78.89; 16.34	73.33; 21.03	$.656_{(19)}$; .520; .295
Stroop – C	53.50; 16.45	55.17; 13.21	$-.264_{(20)}$; .795; .111
Stroop – W&C	37.50; 14.39	33.67; 11.90	$.685_{(20)}$; .502; .290
SRT – LTS	22.90; 11.53	29.50; 15.62	$-1.107_{(20)}$; .282; .480
SRT – CLTR	13.70; 8.87	22.58; 15.77	$-1.581_{(20)}$; .130; .694
SRT – DR	5.90; 1.52	6.42; 3.15	$-.474_{(20)}$; .641; .210
SRT – Intrusions	6.00; 7.54	2.92; 2.47	$1.338_{(20)}$; .196; .549
CERAD – Total hits	18.90; 2.85	20.50; 4.12	$-1.037_{(20)}$; .312; .451
CERAD – DR hits	5.90; 1.37	6.83; 1.80	$-1.345_{(20)}$; .194; .581
MMSE	28.10; 2.28	27.58; 1.78	$.597_{(20)}$; .557; .254
MOCA	20.20; 5.33	22.58; 3.00	$-1.322_{(20)}$; .201; .550
Cognitive dimensions			$t_{(df)}$; *p*; Cohen's d
Executive	.31; .58	.25; .90	$.181_{(19)}$; .858; .079
Memory	-.02; .56	.45; 1.05	$-1.268_{(20)}$; .219; .558

Table 30 - Baseline characteristics on physical functional ability.

Variables (mean, SD)	Non supplem.	Supplem.	
Functional ability – QoFA			$t_{(df)}$; *p*; Cohen's d
Mobility tiredness	4.90; 1.97	3.92; 1.93	$1.180_{(20)}$; .252; .502
Lower limb tiredness	4.60; .52	3.58; 1.44	$2.272_{(14.228)}$; .039; .942
Upper limb tiredness	3.90; .32	3.92; .29	$-.129_{(20)}$; .899; .065
Mobility help	11.80; .63	11.50; .90	$.883_{(20)}$; .388; .386
PADL help	18.20; 4.37	18.50; 2.71	$-.197_{(20)}$; .846; .082
Functional components			$t_{(df)}$; *p*; Cohen's d
Functional tiredness	.30; .49	-.29; .86	$1.923_{(20)}$; .069; .014
Functional help	.13; .89	.11; .77	$.067_{(20)}$; .947; .024

Table 31 – Hematological characterization and comparison at baseline

Variables (mean, SD)	Non supplem.	Supplem.	
Inflammatory indices			$Z_{(U)}$; *p*; *r*
hsCRP (mg/dL) ¥†	1.53; 2.85	2.90; .60	-1.541$_{(37)}$; .134; .329
Red cells indices			$t_{(df)}$; *p*; Cohen's d
RBC (10^{12}/L)	4.49; .50	4.63; .44	-.678$_{(20)}$; .506; .297
Hemoglobin (mg/dL)	13.55; 1.74	12.73; 1.65	1.139$_{(20)}$; .268; .483
Hematocrit (%)	39.51; 4.51	37.85; 3.77	.941$_{(20)}$; .358; .399
MCV (fL)	87.96; 3.32	81.79; 3.52	4.198$_{(20)}$; <.001; 1.803
MCH (pg)	30.12; 1.48	27.43; 1.6	4.071$_{(20)}$; .001; 1.745
MCHC (g/dL)	34.25; .83	33.53; 1.29	1.508$_{(20)}$; .147; .663
RDW (%)	13.51; .60	14.1; 1.14	-1.548$_{(17.252)}$; .140; .647
Iron biomarkers			$t_{(df)}$; *p*; Cohen's d
Fe (µg/dL)	90.80; 27.18	60.17; 21.67	2.944$_{(20)}$; .008; 1.246
TF (mg/dL)	249.10; 28.68	295.08; 53.71	-2.428$_{(20)}$; .025; 1.068
FT (ng/mL) ¥†	67.50; 148.75	19.50; 109.25	-1.814$_{(32.5)}$; .072; .387
TF Sat (%)	28.18; 9.66	16.19; 7.49	3.283$_{(20)}$; .004; 1.388
TIBC (µg/dL)	327.60; 31.48	390.42; 74.34	-2.485$_{(20)}$; .022; 1.100
sTFr (mg/L) ¥†	1.11; .38	1.62; .86	-3.166$_{(12)}$; <.001; .675
sTFr-LogFT index ¥†	.69; .33	1.21; .99	-2.506$_{(22)}$; .011; .534
Body iron (mg/kg)	13.84; 3.64	8.97; 5.54	2.380$_{(20)}$; .027; 1.041
FT class (n; %)			*p*(Fisher exact test; 2 tailed)
FT<45 (ng/mL)	5; 22.72	8; 36.36	.666
FT≥45 (ng/mL)	5; 22.72	4; 18.18	
Iron dimensions			$t_{(df)}$; *p*; Cohen's d
Storage	-.06; .66	-1.32; 1.40	2.031$_{(20)}$; .016; 1.261
Transport	-.03; .96	-1.17; .74	3.170$_{(20)}$; .005; 1.330
Transport S.	.15; .62	-.97; 1.31	2.463$_{(20)}$; 0.023; 1.093
Red cells C.	-.08; .72	-1.43; .98	3.722$_{(19.711)}$; .001; 1.570
Erythropoiesis	-.18; 1.13	-.40; .99	.476$_{(20)}$; .639; .207

¥ Variables not normally distributed, data presented in median and interquartile range (median, IQR); † Mann–Whitney U test, results presented in $Z_{(U)}$; *p*, *r*.

4.2.2 Variation of hematological dimensions during intervention

New component scores for hematological dimensions were calculated for moment A and moment B using the hematological variables and the same methodology as previously described (storage moment A, Cronbach's alpha: .969; storage moment B, Cronbach's alpha: .959; transport moment A, Cronbach's alpha: .976; transport moment B, Cronbach's alpha:.975; transport s. moment A, Cronbach's alpha: .996; transport s. moment B, Cronbach's alpha: .991; red cells c. moment A, Cronbach's alpha: .810; red cells c. moment B, Cronbach's alpha: .916; erythropoiesis moment A , Cronbach's alpha: .964; erythropoiesis moment B, Cronbach's alpha: .935). The new component scores for hematological dimensions obtained for moment A and B were compared using repeated measures ANOVA (Table 32). No significant effects for time or time by group were observed in any of the hematological components. A group effect was observed for storage, transport, transport s., and red cells c.; however, it was not significant for erythropoiesis.

Table 32 - Repeated measures ANOVA for hematological dimensions

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
Storage	.57; .63	-.47; 1,02	.47; .61	-.39; 1,11	$F_{(1; 20)}$=.021; .887; .001
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1; 20)}$=6.268; .021; .239			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1; 20)}$=2.523; .128; .112			
Transport	.62; .95	-.52; .73	.82; .64	-.68; .67	$F_{(1; 20)}$=.010; .923; .000
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1; 20)}$=23.213; <.001; .537			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1; 20)}$=1.165; .293; .055			
Transport S.	.52; .53	-.43; 1,11	.59; .55	-.49; 1,04	$F_{(1; 20)}$=.005; .946; .000
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1; 20)}$=7.870; .011; .282			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1; 20)}$=.559; .463; .027			
Red cells C.	.68; .66	-.64; .89	.52; .39	-.47; 1,16	$F_{(1; 19)}$=.000; .991; .000
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1; 19)}$=12.100; .003; .389			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1; 19)}$=1.190; .289; .059			
Erythropoiesis	.12; 1.09	-.13; .10	.22; 1,03	-.2; .97	$F_{(1; 19)}$=.013; .909; .001
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1; 19)}$=.602; .447; .031			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1; 19)}$=.398; .536; .021			

Regarding the analysis of repeated measures for hematological variables (Table 33) and in consonance with the results of the hematological dimensions, no significant effects (for time, group or time by group interaction) were observed for the variables that composed the erythropoiesis dimension (RBC, hemoglobin and hematocrit). The variables that composed the red cells C. dimension (MCV, MCH, MCHC and RDW) did not reveal a significant effect of time by group interaction, the group effect was significant only for MCH and RDW and MCH and MCHC displayed a significant time effect, not present in MCV and RDW. The variables that composed transport s. (TF and TIBC) and transport (Fe and TF sat.) dimensions did not reveal significant time or time by group interaction effects, although a significant group effect was observed. Regarding the variables that composed the storage dimension, no significant effects were observed for FT (logarithmic transformed), significant time and group effects were observed for sTFR (logarithmic transformed), a significant effect group was observed for sTFr-LogFT index (logarithmic transformed) and a significant interaction of time by group was observed for body iron.

Table 33 - Repeated measures ANOVA for hematological variables.

Variables	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time)
(mean; SD)	Non supplem.	Supplem.	Non supplem.	Supplem.	
RBC (10^{12}/L)	4.49; .50	4.62; .46	4.61; .47	4.61; .39	$F_{(1;19)}$=.583; .454; .030
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=.118; .735; .006			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=.840; .371; .042			
Hemoglobin (mg/dL)	13.55; 1.74	12.65; 1.71	13.82; 1.53	12.69; 1.87	$F_{(1;19)}$=.510; .484; .026
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=1.976; .176; .094			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=.296; .592; .015			
Hematocrit (%)	39.51; 4.51	37.71; 3.93	40.74; 4.07	38.71; 4.02	$F_{(1;19)}$=4.063; .058; .176
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=1.247; .278; .062			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=.043; .838; .002			
MCV (fL)	87.96; 3.32	81.61; 3.63	88.38; 3.28	84.05; 6.66	$F_{(1;19)}$=4.215; .054; .182
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=8.424; .009; .307			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=2.100; .164; .100			
MCH (pg)	30.12; 1.48	27.31; 1.62	29.96; 1.39	27.51; 3.10	$F_{(1;19)}$=.003; .956; .000
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=10.293; 0.005; 0.351			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=0.252; 0.621; 0.013			
MCHC (g/dL)	34.25; .83	33.46; 1.33	33.89; .72	32.65; 1.68	$F_{(1;19)}$=7.741; .012; .289
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=4.241; .053; .182			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=1.142; .299; .057			
RDW (%)	13.51; .61	14.17; 1.17	13.25; .32	14.14; 1.01	$F_{(1;19)}$=.620; .441; .032
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;19)}$=5.674; .028; .230			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;19)}$=.353; .560; .018			
Fe (µg/dL)	90.80; 27.18	60.17; 21.67	102.8; 18.44	62.50; 21.61	$F_{(1;20)}$=1.917; .181; .087
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=19.374; <.001; .492			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.872; .362; .042			
TF (mg/dL)	249.10; 28.68	295.08; 53.71	237.40; 21.50	286.67; 41.59	$F_{(1;20)}$=3.995; .059; .166
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=8.712; .008; .303			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.106; .748; .005			
FT (Log) (ng/dL) [#]	77.59(42.13;142.90)	31.46(13.91;71.16)	78.22(40.15;152.39)	43.68(21.25;89.77)	$F_{(1;20)}$=1.078; .312; .051
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=.924; .348; .044			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.034; .856; .002			
TF Sat. (%)	28.18; 9.66	16.19; 7.49	31.85; 6.41	16.72; 5.83	$F_{(1;20)}$=1.696; .208; .078
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=24.583; <.001; .551			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.939; .344; .045			
TIBC (µg/dL)	327.60; 31.48	390.42; 74.34	325.50; 31.16	377.25; 56.16	$F_{(1;20)}$=1.583; .223; .073
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=6.810; .017; .254			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.832; .373; .040			
sTFr (Log) (mg/L) [#]	1.10(.95;1.27)	1.73(1.37;2.18)	1.25(1.16;1.35)	1.85(1.41;2.42)	$F_{(1;20)}$=5.174; .034; .206
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=7.047; .015; .261			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.064; .803; .003			
sTFr-LogFT index (Log) [#]	.59(.46;.77)	1.23(.80;1.90)	.67(.55;.83)	1.17(.76;1.81)	$F_{(1;20)}$=.012; .915; .001
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=3.985; .060; .166			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=1.601; .220; .074			
Body iron (mg/kg)	13.84; 3.64	8.97; 5.54	13.42; 3.59	9.92; 5.22	$F_{(1;20)}$=.925; .348; .044
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=4.464; .047; .182			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=6.467; .019; .244			

[#] Data presented as geometric mean and back-transformed 95% confidence intervals.

4.2.3 Comparison of pre- and post-intervention differences in cognitive variables

Due to the impossibility to repeat some of the neurocognitive test in a short interval of time, no repeated measures were available from moment A to moment B; however, different measures for the same construct were used in moments A and B. PCA was used to reduce MMSE and MOCA total scores to a dimension termed general cog. (general cognition dimension from MMSE and MOCA, Cronbach's alpha: .782) which measured the same construct as the ACER total score (transformed to a z-score to express the variable in the same scale as the general cog.). MMSE, MOCA and ACER can be divided into sub-scores measuring different areas of cognitive function such as attention, memory, verbal fluency, language and visuospatial orientation. Similar to general cog., PCA was used to reduce the sub-scores of MMSE and MOCA, resulting in 4 dimensions measuring cognitive ability in moment A similar to the sub-scores (as z-scores) of the ACER measured in moment B. Briefly, attention dimension (attention and orientation, Cronbach's alpha: .776) was obtained from the sum of the scores of questions 1 (orientation), 2 (registration) and 3 (attention and calculation) of MMSE and the sum of attention and orientation of MOCA; memory dimension (memory and recall, Cronbach's alpha: .112) as result of PCA of the question 4 (recall) of MMSE and delayed recall question of MOCA; language dimension (language skills, Cronbach's alpha: .610) composed by question 5 (language) of MMSE and language question (first question of language) of MOCA; and a visuospatial dimension (flexibility, planning and conceptualization ability, Cronbach's alpha: .730) composed by question 6 (constructive ability) of MMSE and visuospatial/executive questions of MOCA. Since verbal fluency is not assessed in the MMSE, a verbal fluency domain (phonetic and semantic fluency, Cronbach's alpha: .650) was obtained from PCA of the COWAT-FAS admissible (FAS – Ad.) score and the verbal fluency question (second question of language) of MOCA, which was used also for comparison with verbal fluency sub-scale (z-score) of ACER.

The low reliability (measured by Cronbach's alpha: .112) of the memory dimension prevented us from using this dimension in the analysis. For the remaining dimensions (general cog., attention, verbal fluency, language and visuospatial), no significant differences were observed between non supplemented and supplemented group in moments A or B (Table 34). Due to the use of different tests in each moment (although measuring the same construct), the test of differences between the same group in the different moments or the use of repeated measures ANOVA was not possible.

Table 34 - Neurocognitive pre- and post-intervention comparison of differences.

Variables	Pre-treatment (moment A)			Post-treatment (moment B)		
(mean; SD)	Non supplem.	Supplem.	$t_{(df)}$; *p*; Cohen's d	Non supplem.	Supplem.	$t_{(df)}$; *p*; Cohen's d
General Cog.						
General cog. (MMSE&MOCA)	-.09; 1,25	.07; .79	$-.375_{(20)}$; .712; .168			
Z – ACER total				-.23; 1,15	.19; .86	$-.994_{(20)}$; .330; .449
Attention						
Attention (MMSE&MOCA)	-.28; 1,20	.23; .77	$-1,163_{(14,774)}$; .263; .544			
Z – Attention ACER				-.08; 1,13	.06; .92	$-.317_{(20)}$; .755; .142
Verbal fluency						
Fluency (FAS-Ad.&MOCA)	.08; 1,01	-.07; 1,03	$.330_{(20)}$; .745; .148			
Z – Fluency ACER				-.12; 1,01	.1; 1,03	$-.493_{(20)}$; .627; .221
Language						
Language (MMSE&MOCA)	-.09; 1,14	.08; .91	$-.378_{(20)}$; .709; .170			
Z – Language ACER				-.35; 1,06	.29; .89	$-1,541_{(20)}$; .139; .692
Visio-spatial						
Visio-spatial (MMSE&MOCA)	-.06; 1,14	.05; .91	$-.239_{(20)}$; .814; .107			
Z – Visio-spatial ACER				-.18; .97	.15; 1,04	$-.787_{(19,675)}$; .441; .351

Repeated measures from moment A to moment B were available for BNT-15 and SRT (CLTR, LTS, DR and intrusions) and, therefore, repeated measures ANOVA was used to assess the test time, group and time by group effects on the scores for these tests. In order to reduce multiple comparisons PCA was used to obtain a memory (SRT-PCA) dimension (memory moment A, Cronbach's alpha: .935; memory moment B, Cronbach's alpha: .931) was composed by LTS, CLTR and DR scores (intrusions score was excluded due to low communalities). The standardized value of the BNT-15 was used since different sets of images were used in moments A and B. Statistical analysis was performed using repeated measures ANOVA and is presented in Table 35.

A significant effect of time by group was observed for the BNT-15, meaning that the tendency of non supplemented and supplemented groups was significantly different over intervention. The effects over time and between groups were not statistical significant. In moment A, non supplemented group scored higher than supplemented group, after intervention, in moment B, supplemented group scored higher than non supplemented group. Of notice, since these results are presented in z-scores, an improvement in the values of supplemented group will lead to a decrease in the values of non supplemented group; furthermore, the change in the signal (from positive to negative z-scores in non supplemented group and the other way around in the supplemented group) indicates that the improvement was in a magnitude sufficient to change the position of these groups in relation to the mean value of the variable. Regarding the memory (SRT-PCA) dimension no significant effects for group, time or time by group were observed. Since the use of standardized residuals of the PCA can hide the real difference, the original values that

composed the memory (SRT-PCA) dimension, were used. No significant effects were observed for SRT – LTS, SRT – CLTS AND SRT – DR, which is consistent with the results of the repeated measures ANOVA for the memory (SRT-PCA) dimension.

Table 35 - Repeated measures ANOVA of neurocognitive assessment.

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
BNT-15 (z-score)	.70; .89	-.59; .66	-.05; 1.08	.04; .98	$F_{(1;20)}$=.094; .762; .005
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=3.277; .085; .141			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=11.419; .003; .363			
Memory (SRT-PCA)	-.26; .66	.22; 1.20	-.31; .72	.26; 1.15	$F_{(1;20)}$=.001; .980; .000
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=1.888; .185; .086			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.076; .785; .004			
SRT – LTS	22.90; 11.53	29.50; 15.62	26.50; 10.99	36.42; 13.06	$F_{(1;20)}$=3.868; .063; .162
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=.385; .542; .019			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=2.816; .109; .123			
SRT - CLTR	13.70; 8.87	22.58; 15.77	19.10; 10.27	27.83; 14.90	$F_{(1;20)}$=3.107; .093; .134
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=3.494; .076; .149			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.001; .980; .000			
SRT - DR	5.90;1.52	6.42; 3.15	5.30; 1.77	5.83; 3.43	$F_{(1;20)}$=1.926; .180; .088
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Group)		$F_{(1;20)}$=.243; .628; .012			
$F_{(df1;df2)}$; p; $\eta^2_{partial}$ (Time*group)		$F_{(1;20)}$=.000; .985; .000			

4.2.4 Longitudinal analysis of psychological morbidity difference between groups

Similar to the neurocognitive assessment, no repeated measures were available for neuropsychological evaluation and therefore different measures were used to assess the same construct. For the same reason and as explained for the neurocognitive analysis, the use of repeated measures ANOVA or the test of difference for the same group between the different moments was not possible. Data regarding neuropsychological assessment and test of differences between groups for each moment are presented in Table 36. Values are presented both in original scores and in z-scores allowing for an easier comparison. No significant differences were observed between groups at moments A or B in any of the scales used to assess psychological morbidity.

Table 36 - Pre- and post-intervention comparison of differences for neuropsychological assessment

Variables (mean; SD)	Pre-treatment (moment A)			Post-treatment (moment B)		
	Non supplem.	Supplem.	$t_{(df)}$; *p*; Cohen's d	Non supplem.	Supplem.	$t_{(df)}$; *p*; Cohen's d
Depression						
BDI	7.00; 5.27	5.75; 5.85	$.522_{(20)}$; .608; .234			
Z–BDI	.12; .96	-.10; 1.06				
EADS–D				3.00; 2.40	1.33; 1.44	$2.015_{(20)}$; .058; .905
Z–EADS–D				.44; 1.16	-.37; .69	
Anxiety						
BAI	13.30; 7.42	1.92; 7.27	$.759_{(20)}$; .457; .341			
Z–BAI	.18; 1.02	-.15; 1.00				
EADS–A				2.30; 1.57	3.42; 2.75	$-1.195_{(17.932)}$; .248; .511
Z–EADS–A				-.26; .68	.22; 1.19	
Stress						
PSS	17.70; 7.66	18.58; 6.32	$-.297_{(20)}$; .770; .133			
Z–PSS	-.07; 1.13	.06; .93				
EADS–S				2.70; 2.91	3.25; 2.90	$-.443_{(20)}$; .663; .199
Z–EADS–S				-.11; 1.02	.09; 1.02	

4.2.5 Time-treatment interaction on physical performance and functional ability

The assessment of physical performance consisted in the evaluation of balance evaluation (static balance, gait balance and total; POMA); walking ability (time, steps, gait and cadence; 6MTW), strength (hand and forearm muscular strength; hand grip strength) and functional ability (mobility tiredness, lower limb tiredness, upper limb tiredness, mobility help and physical activities of daily living help; QoFA).

Data and results from repeated measures ANOVA of balance are presented in Table 37. Briefly, significant effects of time were observed only on gait balance. No significant effects of group were observed in any of the assessments performed. The effects of the time by group interaction were significant in all dimensions assessed for balance. In moment A the non supplemented group scored higher than the supplemented group in POMA – static balance, - gait balance and -total; after intervention the mean score of the supplemented group increased in a sufficient magnitude to equal the non supplemented group both in moments A and B. Regarding walking ability (Table 38) no significant effects were observed for group or time by group interaction. For steps and cadence, a significant effect of time was observed. On hand grip strength (Table 39) a significant effect of time by group interaction was observed even though no significant effect was observed for time or group. In moment A the non supplemented group scored higher than the supplemented group; after intervention the score of non supplemented

group decreased when compared to baseline, and that of the supplemented group improved when compared to baseline.

Table 37 - Repeated measures ANOVA for balance

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; η^2 partial (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
POMA – Static balance	14.60; 1.17	13.83; 2.17	14.00; 1.25	14.50; 1.83	$F_{(1;20)}$=.018; .895; .001
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=.038; .847; .002				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=6.467; .019; .244				
POMA – Gait balance	11.60; .97	10.50; 1.57	11.60; .70	11.58; .79	$F_{(1;20)}$=3.667; .070; .155
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=2.325; .143; .104				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=3.667; .070; .155				
POMA – Total	26.20; 1.61	24.33; 3.37	25.60; 1.83	26.08; 2.54	$F_{(1;20)}$=1.627; .217; .075
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=.509; .484; .025				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=6.796; .017; .254				

Table 38 - Repeated measures ANOVA for walking ability

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; η^2 partial (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
6MTW – Time	3.99; .57	4.58; 1.12	3.70; .37	4.62; 1.42	$F_{(1;20)}$=.523; .478; .025
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=3.719; .068; .157				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=.888; .357; .043				
6MTW – Steps	8.03; 1.02	9.19; 1.78	8.73; 1.05	9.45; 1.32	$F_{(1;20)}$=7.587; .012; .275
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=2.852; .107; .125				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=1.699; .207; .078				
6MTW – Gait	91.86; 13.03	82.71; 18.55	98.22; 9.18	84.58; 25.48	$F_{(1;20)}$=2.433; .134; .108
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=2.422; .135; .108				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=.722; .406; .035				
6MTW – Cadence	121.40; 9.29	122.10; 11.68	141.88; 11.75	128.45; 21.23	$F_{(1;20)}$=9.118; .007; .313
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=2.114; .161; .096				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=2.529; .127; .112				

Table 39 - Repeated measures ANOVA for hand grip strength

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; η^2 partial (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
Hand grip strength	29.5; 8.39	30.58; 7.15	27.90; 8.17	31.42; 8.46	$F_{(1;20)}$=.433; .518; .021
$F_{(df1;df2)}$; p; η^2 partial (Group)	$F_{(1;20)}$=.460; .506; .022				
$F_{(df1;df2)}$; p; η^2 partial (Time*group)	$F_{(1;20)}$=4.362; .050; .179				

Similarly to the cross sectional analysis, PCA was used to obtain dimensions of functional ability (functional tiredness and functional help) for each moment (moment A and B). The obtained dimensions were functional-T (functional tiredness moment A, Cronbach's alpha: .341; functional tiredness moment B, Cronbach's alpha: .549) and functional-H (functional help

moment A, Cronbach's alpha: .569; functional help moment B, Cronbach's alpha: .434). The low reliability of the components (Cronbach's alpha <.6) prevented us from using them and therefore each sub-scale of the QoFA was analyzed independently (Table 40). A significant effect of time by group interaction was observed for lower limb tiredness; however, no significant effects of time or group were observed. Once again, the non supplemented group scored higher than supplemented group in moment A; after treatment the mean value of supplemented group was very similar to the mean value of the non supplemented group. In the remaining sub-scales (mobility tiredness, upper limb tiredness, mobility help and PADL help) no significant effects of time, group or time by group interaction were observed.

Table 40 - Repeated measures ANOVA for functional ability

Variables (mean; SD)	Pre-treatment (moment A)		Post-treatment (moment B)		$F_{(df1;df2)}$; p; η^2 partial (Time)
	Non supplem.	Supplem.	Non supplem.	Supplem.	
Mobility tiredness	4.90; 1.97	3.92; 1.93	5.30; .82	5.08; 1.24	$F_{(1;20)}$=3.821; .065; .160
$F_{(df1;df2)}$; p; η^2 partial (Group)		$F_{(1;20)}$=1.231; .280; .058			
$F_{(df1;df2)}$; p; η^2 partial (Time*group)		$F_{(1;20)}$=.915; .350; .044			
Lower limb tiredness	4.60; .52	3.58; 1.44	4.30; .95	4.58; .90	$F_{(1;20)}$=2.048; .168; .093
$F_{(df1;df2)}$; p; η^2 partial (Group)		$F_{(1;20)}$=1.002; .329; .048			
$F_{(df1;df2)}$; p; η^2 partial (Time*group)		$F_{(1;20)}$=7.064; .015; .261			
Upper limb tiredness	3.90; .32	3.92; .29	3.90; .32	3.83; .39	$F_{(1;20)}$=.826; .374; .040
$F_{(df1;df2)}$; p; η^2 partial (Group)		$F_{(1;20)}$=.035; .854; .002			
$F_{(df1;df2)}$; p; η^2 partial (Time*group)		$F_{(1;20)}$=.826; .374; .040			
Mobility help	11.80; .63	11.50; .91	12.00; .00	11.67; .78	$F_{(1;20)}$=.961; .339; .046
$F_{(df1;df2)}$; p; η^2 partial (Group)		$F_{(1;20)}$=1.881; .185; .086			
$F_{(df1;df2)}$; p; η^2 partial (Time*group)		$F_{(1;20)}$=.008; .930; .000			
PADL help	18.20; 4.37	18.50; 2.71	18.40; 3.75	18.67; 2.31	$F_{(1;20)}$=.630; .437; .031
$F_{(df1;df2)}$; p; η^2 partial (Group)		$F_{(1;20)}$=.041; .841; .002			
$F_{(df1;df2)}$; p; η^2 partial (Time*group)		$F_{(1;20)}$=.005; .943; .000			

5.Discussion and conclusions

For a long time it has been known that iron homeostasis is important for health and that imbalances of this tightly regulated metabolism leads to adverse effects on several mechanisms (Nancy C. Andrews, 1999). Specifically, low iron status or iron deficiency, which is the focus of this work, has been associated with negative outcomes in cognitive performance, depression, behavior and physical capacity (J. L. Beard & Connor, 2003; Haas & Brownlie, 2001; Laura E. Murray-Kolb, 2011). Despite of this well-known effect on these domains of human health, the main body of research regarding iron deficiency has been conducted in infants, children and women of child bearing age. However, older individuals are particularly susceptible of being at risk of iron deficiency. Furthermore, due to the aging process, they are more likely to develop cognitive deficits (Deary et al., 2009), depressive mood (Jamison et al., 2006) and physical disabilities (Janssen et al., 2002). Still, little is known about correlates of low iron status in the aging process.

In this work, we examined the associations of normal and lower iron status with cognitive performance, neuropsychological morbidity, functional ability and physical performances in older individuals. In order to explore and understand these associations, we used two distinct approaches: (i) an observational approach, with a cross-sectional design, and (ii) an exploratory approach, using a quasi-experimental study design with iron-based fortification intervention component. Three main objectives served as the guiding thread to the work here addressed. First, using a cross-sectional methodology we investigated the associations of iron deficiency and low iron status with cognition, mood and physical functional ability. Second, we investigated whether iron supplementation ameliorated the iron status. And, third, whether fortification improved neurocognitive and physical performance.

5.1.   Strengths and limitations

The novelty of this work deserves to be highlighted. The population-based observational study is the first work that examines the associations of iron deficiency with cognitive function, neuropsychological morbidity and physical functional ability in older community dwellers. Furthermore, both hematological and neurocognitive assessments were very comprehensive which allowed us to detect association(s) between specific dimensions of iron status (such as storage) and specific dimensions of cognition (such as memory). Since this work was supported by a larger study addressing predictors of healthy cognitive aging, several measures were available to correlate with iron status and the extensive characterization of participants allowed

controlling the main confounding factors that could confound and/or act as effectors in the associations under analysis. The richness of available data and the statistical approach should be highlighted as strengths of this work.

Some limitations of this work should also be addressed. The cross-sectional nature of observational study limits conclusions on the nature of the association between low iron status and the dependent variables addressed. The sample size can also be a limitation. Although it was sufficient to allow the observation of significant associations, it could be not enough to detect more subtle associations in other variables, especially in case of small effect sizes. As such, effect sizes were reported throughout the work. Furthermore, the low prevalence of anemic individuals in our sample did not allow to explore the differential association of iron deficiency, iron deficiency anemia, anemia and normal iron nutriture. In order to overcome these limitations (sample size and conditions prevalence) a larger population-based study should be used. As for all the observational studies, here also, no causality relationship can be established for each association found. This limitation, inherent to the nature of the design, could be overcome using a randomized controlled trial, preferentially double (or even) triple blind. However, due to several practical limitations (including, chiefly ethical constrains), a double blind randomized controlled trial was not performed. Instead a quasi-experimental study design, here designated as intervention study, was used, which complemented the observational study.

In the intervention study the sample size is, in fact, the major limitation although, the nature of the design, which include repeated measures, allows the use of small samples to detect significant differences. Furthermore, the distribution of subjects by branch can also represent a bias. The two branches had similar number of individuals classified as low FT and if, in theory, only the low FT [which are undoubtedly iron deficient (G. H. Guyatt et al., 1992) benefit from iron fortification, the improvements may be diluted by the ones that did not improve for being false iron deficient. Perhaps other approaches should be considered in which all individuals are fortified and results will be compared between the ones that benefit from the intervention, measured by hemoglobin and/or FT response (Mei et al., 2005), and the ones that do not benefit.

Finally, novelty was also one of the features of the intervention study. To our knowledge, the present work was the first one to test the effects of iron fortification on iron status of older individuals, and further address if this strategy correlates with (positive) effects in cognition,

neuropsychological morbidity, functional ability and physical performance. Furthermore, it was also the first that used iron food fortification as a vehicle for iron delivery.

5.2. Iron deficiency or low iron status – What matters?

The participants enrolled in this study were cognitively "healthy" community dwellers (no diagnosed cognitive impairment, dementia and/or neuropsychiatric disorder), whom were clinically considered as "normal/healthy" agers, particularly considering absence of neuropathology, and/or central nervous system disease and/or overt thyroid pathology. Nevertheless, despite of this, subjects with conditions that could affect iron status (chronic kidney disease, malnutrition, inflammatory states and suspects of iron overload) were also further excluded from the analysis. Although iron status is a continuum from iron overload to iron deficiency, our aim was only to address the associations of iron deficiency or low iron status and for that reason, subjects with suspects of iron overload were excluded.

Individuals were classified as iron deficient using a very complete panel of iron biomarkers and according the values proposed in the scientific literature (see Table 1). Since inflammation influences the levels of several of the iron-status biomarkers, all the cases of inflammatory states were excluded (hsCRP>10mg/dL). Even having excluding inflammatory states, a significant difference in the value of hsCRP between iron sufficiency cases and ID cases was observed and, not surprisingly, higher values of hsCRP were observed in the ID group. As expected, the ID group presented significantly different values in the red cells indices, in the iron biomarkers and in the hematological dimensions, without exceptions. Coherently, all the differences indicate a significantly lower iron status of the ID group. The occurrence of hypoferremia following infection (which leads to inflammation) has been recognized for more than a half of century (Thurnham & McCabe, 2012). Furthermore, it is known that inflammatory hallmarks are present in the etiology of ACD and that this relation is mediated by hepcidin, which is over expressed in inflammatory states and negatively regulates iron availability (T. Ganz & Nemeth, 2006; Hentze et al., 2010). Here we classify as iron deficient a group of individuals with a higher inflammatory profile and for that reason we cannot conclude if we are in the presence of an absolute or functional iron deficiency (Thomas et al., 2013).

In order to exclude the inflammation effects on iron status and its associations with the target variables (cognitive, psychological and physical functional ability), all the analyses were controlled for hsCRP levels. Additionally to the hsCRP, all the analyses were controlled for other

main confounding factors. The analysis of neurocognitive and neuropsychological variables was controlled for age, gender and education; GDS score was also used in the analysis of neurocognitive variables, following previous observation in similar cohorts (Nadine Correia Santos et al., 2014). Physical ability variables were also controlled for age and BMI. This strategy gives us a stronger confidence in the results obtained. We used ANCOVA to test whether the iron deficiency classification could be a predictor of the dependent variables and no significant results were observed in any of them, which was unexpected since in other types of population (namely children, adolescents and childbearing age women) iron deficiency was associated with impairments on cognition, mood and physical performance (J. L. Beard et al., 2005; Bodnar, Cogswell, & McDonald, 2005; Grantham-McGregor & Ani, 2001; Halterman, Kaczorowski, Aligne, Auinger, & Szilagyi, 2001). The minimum value of hemoglobin found in our sample was 9.3 mg/dL, where it is known that severe ID will lead to deficient erythropoiesis and therefore to anemia, which will be severe in the same proportion as the ID. The inefficiency of the classification of ID to predict the target variables may be explained by the fact that, in our sample, ID was not sufficiently severe.

Iron status is a continuum and no clear cutoff exists between normal iron status and iron deficiency. In order to test the association of iron status/dimensions with neurocognition, neuropsychological morbidity and physical functional ability hierarchical regression models were constructed. Our data indicate that, even after controlling for the main confounding factors, iron status is associated with memory dimension, GDS and the functional tiredness dimension.

In the same line as previously proposed (J. L. Beard, 2001), and taken together our results, these findings suggest that, from the point of view of cognitive function, mood and physical ability, classifying individuals as iron deficient or non deficient is not a biological reality, since none of the variables taken as dependent was different between iron-deficient and iron-sufficient individuals. Instead considering iron status/nutriture as a continuum seems more accurate and reliable to assess the consequences of different levels of severity.

5.3. Nutritional status and iron nutriture – obviously!

It was previously proposed that an iron deficient state could be a surrogate marker for malnutrition (Fairweather-Tait et al., 2013; Hsu et al., 2013). Although the MNA test was designed to detect protein-energy malnutrition (Guigoz, 2006), some authors (Murphy, Brooks,

New, & Lumbers, 2000) found significantly lower intakes of iron in malnourished orthopedic patients when compared with well-nourished patients. These differences were not corroborated by a study in institutionalized older individuals (Ruiz-López et al., 2003). Using the MNA score as a continuum, a significant correlation was found with iron intake in hospitalized patients. Even though, in theory, MNA scores can be associated with iron status, only a small study so far, to our knowledge, has investigated this (Langkamp-Henken, Hudgens, Stechmiller, & Herrlinger-Garcia, 2005). This study reported a positive association of MNA with hematocrit and hemoglobin in pressure ulcers patients, but they included individuals that ranged from undernourished to normal nutritional status. In our population, excluding undernourished individuals, we observed that ID states can be significantly predicted by MNA. Nutritional status is a predictor of ID and for a long time is a known factor associated to cognition (Goodwin, Goodwin, & Garry, 1983), mood (Boult, Krinke, Urdangarin, & Skarin, 1999) and physical performance (Olin, Koochek, Ljungqvist, & Cederholm, 2004). Taken together, these associations led us to hypothesize that nutritional status could be a mediator of the association of iron status with the target variables. In this study, our hypothesis revealed to be true regarding a mediation effect in the association of storage dimension with memory dimension and will be further discussed.

5.4.   Memory - the neurocognitive facet of lower iron status

From the literature it is clear that the focus of low iron status research and cognition has been based on the categorical classification of iron deficiency, particularly in children and/or in animal models (Fretham et al., 2011) or in anemic individuals (Deal, Carlson, Xue, Fried, & Chaves, 2009; Denny et al., 2006; Lucca et al., 2008; Terekeci et al., 2010). In this work, as previously discussed, classification as iron deficient was not sensitive to discriminate cognitive performance of subjects. From the 151 enrolled subjects only 23 were anemic and from these only 8 were also iron deficient (data not shown). The discrepancy between the number of anemic subjects in each possible condition (unknown=2; iron deficient=36; iron deficiency anemia=8; anemic=15; normal=90) prevented us to conduct an analysis regarding anemia or iron deficiency anemia.

Classification of anemia is made with base in the hemoglobin levels and since it was not possible to perform an analysis with respect to that classification we used hemoglobin levels to construct the erythropoiesys dimension along with hematocrit and RBC. Theoretically, the

associations found with this dimension should be similar, and in the same direction, to the differences found between anemic and non anemic individuals when analyzing similar constructs. The results of hierarchical regression models indicate that the erythropoiesys dimension predicted the memory dimension but did not predict the executive dimension, MMSE or MOCA. It is puzzling that no significant predictive value was observed for the above mentioned variables since several studies reported differences in the performance of these tests (or other tests measuring the same cognitive functions) among anemic and non-anemic older individuals (Deal et al., 2009; Lucca et al., 2008; Terekeci et al., 2010). The absence of statistical significant results can probably be explained by the different methodological approaches and/or it is possible that we do not have sufficient sample size to observe subtle associations. Still, the sample size was sufficient to detect the association of erythropoiesys dimension and memory, indicating perhaps that the magnitude of the possible undetected association is smaller for the executive dimension, MMSE or MOCA than for memory dimension. With respect to the predictive value of the erythropoiesys for memory, our observation is consistent with that by Shah *et al.* (R. C. Shah et al., 2009) who reported lower memory ability in individuals with lower levels of hemoglobin. Similar to our methodological approach the authors considered that the use of hemoglobin as a continuous variable may be a better way to characterize its relationship with cognitive function.

The neurocognitive dimensions/variables were not predicted by any of the remaining hematological variables with exception for the storage dimension which was a predictor of the memory dimension. Once again, our results do not fit with previous reports in which an association between ID and MMSE was observed (Yavuz et al., 2012). In other population groups, namely young women, iron status has been associated with executive function and cognition but in some case this associations are found between the time to complete a task and not the result of the task (Blanton, Green, & Kretsch, 2013; L. E. Murray-Kolb & Beard, 2007). The neurocognitive tests that we used have a defined duration to be completed and are considered finished after a defined number of mistakes, or do not take time into consideration. Several hypotheses can be put forward to explain the discrepancy of results between our study and some previous reports but sample size and especially methodological approach are again the most probable.

Despite of the conflicting results of low iron status and cognitive performance, the observations for memory and storage are consistent with previous observations in children,

adolescents and pregnant women (Fretham et al., 2011; Gordon, 2003; Groner, Holtzman, Charney, & Mellits, 1986). Insights from experiments on animal models of iron deficiency provide clues that possibly explain this interesting finding. The hippocampus is the central processing area for memory (Squire, 2004) and alterations of hippocampus neurochemical profile of rats were observed during perinatal ID which causes neurochemical alterations and possibly persistent changes in the resting energy status, neurotransmission and myelination (Rao, Tkac, Townsend, Gruetter, & Georgieff, 2003). Tran *et al.* (Tran, Fretham, Carlson, & Georgieff, 2009) showed that fetal-neonatal ID lowers hippocampal brain-derived neurotrophic factor expression and function even in adult age. These findings are relevant to the understanding of the molecular basis of the reported association; however, they concern to a period of the brain development in which iron is paramount (Roskams & Connor, 1994). During aging process a certain degree of brain iron accumulation is expected, but its effects are deleterious (Tracey A. Rouault & Cooperman, 2006). Since no studies were published regarding these mechanisms, both in humans and animal models, here we can only suggest the existence of similar mechanisms during iron deficiency in aging.

Since, in the absence of parenteral iron administration dietary iron is the only source of iron (Miret et al., 2003), and nutritional status is associated with cognitive function, depressive mood and physical ability of older individuals (Goodwin et al., 1983; Stuck et al., 1999), we hypothesized that the association of iron status and the dependent variables under analysis could be mediated by nutritional status. Here the only interaction with significant predictive value was storage on memory and it was positive, which means that for individuals with higher stores of iron, if in a good nutritional status, memory function will be at a potential maximum. This finding is particularly interesting and to our knowledge is reported for the first time. It is possible that higher levels of insufficiency are necessary to impact on several of the dimensions studied, and therefore our sample may lack power to detect them.

Interestingly, from the hematological dimensions constructed with iron biomarkers (storage, transport and transport s.) only the storage dimension, alone or in interaction with the nutritional status, was a significant predictor of memory. Stored iron, assessed using storage dimension as a proxy, is readily available when and where it is needed (Linder, 2013), which may justify its impact on memory function.

5.5.   Depressive mood in depressed iron status

The analysis of the predictive value of iron related hematological dimensions on neuropsychological variables revealed that only depressive mood could be predicted. Specifically, erythropoiesis, transport s. and transport dimensions were significant predictors of GDS score. Regarding the erythropoiesis dimension, hemoglobin is one of the principal components and therefore is expected that our results follow the same direction of the reports exploring the associations of anemia and depression. Despite of the differences in the methodological approach our results are in accordance with the results of three previous studies examining the association of anemia and depressive mood. In the InChianti study (Onder et al., 2005) a higher prevalence of anemia was observed among the individuals displaying depressive symptoms. In the same line, the results of the English longitudinal study of ageing (Hamer & Molloy, 2009) showed that, at baseline, anemia was associated with depressive mood, although it was not a predictor of its incidence at a two years follow-up. More recently, Stewart *et al.* (R. Stewart & Hirani, 2012), in a study examining the associations between anemia and iron status with depressive symptoms, found anemia to be associated with depressive mood, although it was reflecting primarily the anemia of chronic disease. This result should be interpreted with caution, since inflammation is a component of anemia of chronic disease and it is associated with depression *per se* (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Altogether, these studies suggest that altered brain oxygenation and dopaminergic function can underlie the observed mood outcome, given the well described involvement of monoamines imbalance in the etiology of depression (Meyer et al., 2006). Other neurotransmitter systems are likely also involved, namely serotoninergic and noradrenergic (Burhans et al., 2005).

5.6.   If we are not made of iron, we get tired

The amount of literature reporting lower physical functioning in anemic older individuals is convincing (Chaves, 2008; Penninx et al., 2003; Penninx et al., 2004; M. Thein et al., 2009). In the present work we observed that both erythropoiesis and red cells c. dimensions were associated with the tiredness dimension of physical functional ability. The logical explanation for the higher fatigability observed in individuals with lower scores in these hematological dimension is the lower oxygenation of the muscles (J. L. Beard, 2001; Penninx et al., 2003).

Literature in the study of iron deficiency and physical ability in older subjects is surprisingly scarce, contrary to the large amount of literature examining the same in anemia. We report a significant predictive value of storage and transport dimensions on the functional-T dimension. Ours is the first report on these aspects in the older population, and seems to be in accordance with reports on other populations, such as hospitalized anemic patients and several physical workers from both sexes (Haas & Brownlie, 2001). Of notice, in iron-depleted, nonanemic women iron supplementation was associated with a significant improvement in muscle fatigability, which can be interpreted as an indication of higher fatigability due to low iron status (Brutsaert et al., 2003).

5.7.   Can we reverse it and its liabilities?

Using a quasi-experimental design we tested the hypothesis of cognitive function, mood and physical performance amelioration or normalization with daily iron food fortification with low iron dosage.

From the hematological dimensions/variables, with exception of body iron, no significant effects of the intervention were observed. Rimon *et al.* (Rimon et al., 2005) reported an increase in hemoglobin and ferritin concentrations in iron deficient anemic elderly treated with a daily dosage of 15 mg of iron during 60 days. Although the dosages in our study and theirs were similar and the duration of our intervention was greater we did not observed the same findings. Several differences should be considered. In their work the subjects were iron deficient anemic and therefore more prompt to absorb iron and to have a greater amelioration of hematological parameters. Also, different formulations of iron were used; however, $Na(Fe^{3+})EDTA$ should be better absorbed than the liquid ferrous gluconate they used (Dary & Hurrell, 2006). Despite of no significant effects observed in almost all the hematological variables, body iron significantly increased (significant effect of time by group interaction) from pre-treatment to post treatment in the supplemented group which could have implications for the results that will be discussed further ahead.

Here neurocognitive and neuropsychological variables were not different between the two time points and/or as a result of the intervention. Results from randomized controlled trials indicate that iron supplementation is capable of ameliorating or normalizing neurocognition (Falkingham et al., 2010) and neuropsychological morbidity (Laura E. Murray-Kolb, 2011) in children and adults. The iron dosage used in these studies was higher than the one that we used

and it is possible that our iron dosage is insufficient to promote a visible amelioration. The BNT-15 score was significantly improved by the intervention, although it is possible that this result is more dependent on the engagement of the individuals on the fortification branch than as a result of the iron fortification.

The main findings on the longitudinal study are the improvement on the balance (POMA – static balance, POMA – gait balance and POMA – total), on the hand grip strength and the reduction in lower limb tiredness. These observations are consistent with the hypothesis that iron contributes to physical performance. Anemia has been associated with an increased risk of falls (Dharmarajan, Avula, & Norkus, 2007; Mei Sheng et al., 2008) and if the type of association is causal, the treatment of anemia will result in a reduced risk of falls. Here we observed an improvement in the scores of POMA, which is used to assess the risk of falls in older individuals (Tinetti, 1986).

Hand grip strength and lower limb tiredness improved with supplementation, which may reflect endurance and aerobic capacity. Iron deficiency, particularly iron deficiency anemia was associated with a compromised aerobic and endurance capacity in a large number of studies from animal to humans and it was hypothesized that, further to deficient oxygen delivery, tissue iron deficiency may also play a role through reduced cellular oxidative capacity (Haas & Brownlie, 2001).

5.8.   Concluding remarks and future directions

Iron deficiency is the most common nutritional deficiency in the world and older individuals are particular susceptible to developed negative imbalances in iron homeostasis, particularly if a pathological condition is present. In an aging society, efforts to understand and identify issues that can be used to improve or maintain health and wellbeing of agers are paramount. Cognition and physical functioning are particularly relevant to maintain independence in late life. Thus, the study and investigation of strategies for a healthy aging is particularly relevant, not only given the health and social benefits, but also by the reduction of the expenditure of the health and welfare systems of nations.

Here, using complementary study designs, we demonstrated an association of the lower half of the iron status range and memory, depressive mood and physical functional tiredness in older community dwellers. These finding are particularly relevant if we take into account the dimension of the public health problem that is ID and that it impacts essential capabilities of

aging individuals. The traditional viewpoint that iron deficiency does not have consequences until the development of anemia is challenged here, since our sample had a very low number of anemic individuals.

The observations from the longitudinal study indicate that physical performance and ability probably can be ameliorated with nutritional interventions of iron fortified foods.

In conclusion, low iron status of older individuals is associated with more depressive mood, higher tiredness from physical activities of daily living and lower memory, which seem to be modulated by nutritional status. Further research is needed to replicate and confirm our results, as well as, insights from animal models of low iron status and aging, which can be an advantage to understand the mechanistic aspects that underlie these observations.

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